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(54) Title: ANTISENSE MODULATION OF LIVER GLYCOGEN PHOSPHORYLASE EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of liver glycogen phosphorylase. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding liver glycogen phosphorylase. Methods of using these compounds for modulation of liver glycogen phosphorylase expression and for treatment of diseases associated with expression of liver glycogen phosphorylase are provided.

ANTISENSE MODULATION OF LIVER GLYCOGEN PHOSPHORYLASE EXPRESSION

FIELD OF THE INVENTION

5   The present invention provides compositions and methods for modulating the expression of liver glycogen phosphorylase. In particular, this invention relates to antisense compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding human
10   liver glycogen phosphorylase. Such oligonucleotides have been shown to modulate the expression of liver glycogen phosphorylase.

BACKGROUND OF THE INVENTION

There are several mechanisms in place for the
15   homeostatic regulation of biological processes, and balanced energy metabolism is critical to all of these mechanisms. In higher organisms energy stores are in the form of glycogen and upon energy deficit these stores are mobilized through enzymatic digestion to glucose-1-
20   phosphate by the glycogen phosphorylase family of proteins.

Mammalian glycogen phosphorylases comprise a family of three isozymes which are distinguished by their electrophoretic mobilities, immunological properties and tissue-specific distribution. Each isozyme is encoded by a
25   different gene, and these genes have been denoted PYGL, PYGM and PYGB, for liver, muscle and brain isoforms, respectively (Newgard et al., *Crit. Rev. Biochem. Mol. Biol.*, **1989**, *24*, 69-99). The primary control however, common to all isozymes, is the phosphorylation of the
30   inactive state, (b), to the active phosphorylated state, (a). This phosphorylation on serine-14 stabilizes the subunits of the homodimer and alters the binding sites for allosteric effectors and substrates (Sprang et al., *Nature*, **1988**, *336*, 215-221).

Liver glycogen phosphorylase (also known as 1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, glycogen phosphorylase (liver), EC 2.4.1.1 and HLGPa, for human liver glycogen phosphorylase a) is the enzyme which
5 catalyzes the degradation of stored glycogen in the liver to glucose-1-phosphate by the cleavage of α-1,4-glycosidic bonds and therefore plays a critical role in carbohydrate metabolism and blood glucose homeostasis (Newgard et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1986**, *83*, 8132-8136). The
10 activity of liver glycogen phosphorylase is tightly regulated requiring the presence of a cofactor, pyridoxal phosphate, and involving allosteric mechanisms which include activation by AMP and glycogen binding and inhibition by glucose and glucose-6-phosphate binding. The
15 enzyme is also regulated through phosphorylation by phosphorylase kinase which activates the homodimer (Keppens et al., *Hepatology,* **1993**, *17*, 610-614).

The gene for liver glycogen phosphorylase (PYGL) has been mapped to chromosome 14 and mutations in this gene
20 give rise to glycogen storage disease type VI (GSD VI) or Hers Disease, a group of disorders that cause hepatomegaly and hypoglycemia (Burwinkel et al., *Am. J. Hum. Genet.,* **1998**, *62*, 785-791; Chang et al., *Hum. Mol. Genet.,* **1998**, *7*, 865-870; Newgard et al., *Am. J. Hum. Genet.,* **1987**, *40*, 351-
25 364). These mutations consist of two splice-site mutations which result in aberrant exon retention and exon skipping and two missense mutations which produce nonconservative replacements of amino acids that are normally conserved in all eukaryotes.
30 To date, two types of inhibitors targeting glycogen phosphorylase function have been reported. These involved the use of glucose analogs containing multiple polar groups which bind near the active site of the protein (Lundgren et al., **1997**; Lundgren and Kirk, **1995**) and caffeine and other

heteroaromatic analogs which bind at the purine inhibitory
site (Kasvinsky et al., *Can. J. Biochem.*, **1981**, *59*, 387-
395; Kasvinsky et al., *J. Biol. Chem.*, **1978**, *253*, 3343-
3351; Kasvinsky et al., *J. Biol. Chem.*, **1978**, *253*, 9102-
5 9106). However, none of these compounds have been shown to
be orally active, limiting their utility.

Recently, the discovery of an orally active compound,
CP-91149 and derivatives thereof, that lowers plasma
glucose levels in an animal model of type 2 diabetes was
10 reported (Hoover et al., *J. Med. Chem.*, **1998**, *41*, 2934-
2938; Martin et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1998**,
95, 1776-1781). This indole-containing compound was shown
to inhibit glycogenolysis in diabetic ob/ob mice, and in
rat and human liver cells by inhibiting liver liver
15 glycogen phosphorylase. It is believed that inhibition of
glycogenolysis will be of therapeutic benefit in the
treatment of diabetes, particularly type II diabetes.

Antisense technology is emerging as an effective means
for reducing the expression of specific gene products and
20 may therefore prove to be uniquely useful in a number of
therapeutic, diagnostic, and research applications for the
modulation of glycogen phosphorylase expression.

SUMMARY OF THE INVENTION

The present invention is directed to antisense
25 compounds, particularly oligonucleotides, which are
targeted to a nucleic acid encoding liver glycogen
phosphorylase, and which modulate the expression of liver
glycogen phosphorylase. Pharmaceutical and other
compositions comprising the antisense compounds of the
30 invention are also provided. Further provided are methods
of modulating the expression of liver glycogen
phosphorylase in cells or tissues comprising contacting
said cells or tissues with one or more of the antisense
compounds or compositions of the invention. Further

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provided are methods of treating an animal, particularly a
human, suspected of having or being prone to a disease or
condition associated with expression of liver glycogen
phosphorylase by administering a therapeutically or
5 prophylactically effective amount of one or more of the
antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

    The present invention employs oligomeric antisense
compounds, particularly oligonucleotides, for use in
10 modulating the function of nucleic acid molecules encoding
liver glycogen phosphorylase, ultimately modulating the
amount of liver glycogen phosphorylase produced. This is
accomplished by providing antisense compounds which
specifically hybridize with one or more nucleic acids
15 encoding liver glycogen phosphorylase. As used herein, the
terms "target nucleic acid" and "nucleic acid encoding
liver glycogen phosphorylase" encompass DNA encoding liver
glycogen phosphorylase, RNA (including pre-mRNA and mRNA)
transcribed from such DNA, and also cDNA derived from such
20 RNA. The specific hybridization of an oligomeric compound
with its target nucleic acid interferes with the normal
function of the nucleic acid. This modulation of function
of a target nucleic acid by compounds which specifically
hybridize to it is generally referred to as "antisense".
25 The functions of DNA to be interfered with include
replication and transcription. The functions of RNA to be
interfered with include all vital functions such as, for
example, translocation of the RNA to the site of protein
translation, translation of protein from the RNA, splicing
30 of the RNA to yield one or more mRNA species, and catalytic
activity which may be engaged in or facilitated by the RNA.
The overall effect of such interference with target nucleic
acid function is modulation of the expression of liver
glycogen phosphorylase. In the context of the present
35 invention, "modulation" means either an increase

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(stimulation) or a decrease (inhibition) in the expression
of a gene. In the context of the present invention,
inhibition is the preferred form of modulation of gene
expression and mRNA is a preferred target.

5   It is preferred to target specific nucleic acids for
antisense. "Targeting" an antisense compound to a
particular nucleic acid, in the context of this invention,
is a multistep process. The process usually begins with
the identification of a nucleic acid sequence whose
10 function is to be modulated. This may be, for example, a
cellular gene (or mRNA transcribed from the gene) whose
expression is associated with a particular disorder or
disease state, or a nucleic acid molecule from an
infectious agent. In the present invention, the target is
15 a nucleic acid molecule encoding liver glycogen
phosphorylase. The targeting process also includes
determination of a site or sites within this gene for the
antisense interaction to occur such that the desired
effect, e.g., detection or modulation of expression of the
20 protein, will result. Within the context of the present
invention, a preferred intragenic site is the region
encompassing the translation initiation or termination
codon of the open reading frame (ORF) of the gene. Since,
as is known in the art, the translation initiation codon is
25 typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in
the corresponding DNA molecule), the translation initiation
codon is also referred to as the "AUG codon," the "start
codon" or the "AUG start codon". A minority of genes have
a translation initiation codon having the RNA sequence
30 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG
have been shown to function in vivo. Thus, the terms
"translation initiation codon" and "start codon" can
encompass many codon sequences, even though the initiator
amino acid in each instance is typically methionine (in
35 eukaryotes) or formylmethionine (in prokaryotes). It is

also known in the art that eukaryotic and prokaryotic genes
may have two or more alternative start codons, any one of
which may be preferentially utilized for translation
initiation in a particular cell type or tissue, or under a
5 particular set of conditions. In the context of the
invention, "start codon" and "translation initiation codon"
refer to the codon or codons that are used in vivo to
initiate translation of an mRNA molecule transcribed from a
gene encoding liver glycogen phosphorylase, regardless of
10 the sequence(s) of such codons.

It is also known in the art that a translation
termination codon (or "stop codon") of a gene may have one
of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the
corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA,
15 respectively). The terms "start codon region" and
"translation initiation codon region" refer to a portion of
such an mRNA or gene that encompasses from about 25 to
about 50 contiguous nucleotides in either direction (i.e.,
5' or 3') from a translation initiation codon. Similarly,
20 the terms "stop codon region" and "translation termination
codon region" refer to a portion of such an mRNA or gene
that encompasses from about 25 to about 50 contiguous
nucleotides in either direction (i.e., 5' or 3') from a
translation termination codon.

25   The open reading frame (ORF) or "coding region," which
is known in the art to refer to the region between the
translation initiation codon and the translation
termination codon, is also a region which may be targeted
effectively. Other target regions include the 5'
30 untranslated region (5'UTR), known in the art to refer to
the portion of an mRNA in the 5' direction from the
translation initiation codon, and thus including
nucleotides between the 5' cap site and the translation
initiation codon of an mRNA or corresponding nucleotides on
35 the gene, and the 3' untranslated region (3'UTR), known in

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the art to refer to the portion of an mRNA in the 3'
direction from the translation termination codon, and thus
including nucleotides between the translation termination
codon and 3' end of an mRNA or corresponding nucleotides on
5   the gene. The 5' cap of an mRNA comprises an N7-methylated
guanosine residue joined to the 5'-most residue of the mRNA
via a 5'-5' triphosphate linkage. The 5' cap region of an
mRNA is considered to include the 5' cap structure itself
as well as the first 50 nucleotides adjacent to the cap.
10 The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly
translated, many contain one or more regions, known as
"introns," which are excised from a transcript before it is
translated. The remaining (and therefore translated)
15 regions are known as "exons" and are spliced together to
form a continuous mRNA sequence. mRNA splice sites, i.e.,
intron-exon junctions, may also be preferred target
regions, and are particularly useful in situations where
aberrant splicing is implicated in disease, or where an
20 overproduction of a particular mRNA splice product is
implicated in disease. Aberrant fusion junctions due to
rearrangements or deletions are also preferred targets. It
has also been found that introns can also be effective, and
therefore preferred, target regions for antisense compounds
25 targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified,
oligonucleotides are chosen which are sufficiently
complementary to the target, i.e., hybridize sufficiently
well and with sufficient specificity, to give the desired
30 effect.

In the context of this invention, "hybridization" means
hydrogen bonding, which may be Watson-Crick, Hoogsteen or
reversed Hoogsteen hydrogen bonding, between complementary
nucleoside or nucleotide bases. For example, adenine and
35 thymine are complementary nucleobases which pair through

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the formation of hydrogen bonds. "Complementary," as used
herein, refers to the capacity for precise pairing between
two nucleotides. For example, if a nucleotide at a certain
position of an oligonucleotide is capable of hydrogen
5   bonding with a nucleotide at the same position of a DNA or
RNA molecule, then the oligonucleotide and the DNA or RNA
are considered to be complementary to each other at that
position. The oligonucleotide and the DNA or RNA are
complementary to each other when a sufficient number of
10 corresponding positions in each molecule are occupied by
nucleotides which can hydrogen bond with each other. Thus,
"specifically hybridizable" and "complementary" are terms
which are used to indicate a sufficient degree of
complementarity or precise pairing such that stable and
15 specific binding occurs between the oligonucleotide and the
DNA or RNA target. It is understood in the art that the
sequence of an antisense compound need not be 100%
complementary to that of its target nucleic acid to be
specifically hybridizable. An antisense compound is
20 specifically hybridizable when binding of the compound to
the target DNA or RNA molecule interferes with the normal
function of the target DNA or RNA to cause a loss of
utility, and there is a sufficient degree of
complementarity to avoid non-specific binding of the
25 antisense compound to non-target sequences under conditions
in which specific binding is desired, i.e., under
physiological conditions in the case of in vivo assays or
therapeutic treatment, and in the case of in vitro assays,
under conditions in which the assays are performed.
30   Antisense compounds are commonly used as research
reagents and diagnostics. For example, antisense
oligonucleotides, which are able to inhibit gene expression
with exquisite specificity, are often used by those of
ordinary skill to elucidate the function of particular
35 genes. Antisense compounds are also used, for example, to

distinguish between functions of various members of a
biological pathway. Antisense modulation has, therefore,
been harnessed for research use.

The specificity and sensitivity of antisense is also
5 harnessed by those of skill in the art for therapeutic
uses. Antisense oligonucleotides have been employed as
therapeutic moieties in the treatment of disease states in
animals and man. Antisense oligonucleotides have been
safely and effectively administered to humans and numerous
10 clinical trials are presently underway. It is thus
established that oligonucleotides can be useful therapeutic
modalities that can be configured to be useful in treatment
regimes for treatment of cells, tissues and animals,
especially humans.
15 In the context of this invention, the term
"oligonucleotide" refers to an oligomer or polymer of
ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or
mimetics thereof. This term includes oligonucleotides
composed of naturally-occurring nucleobases, sugars and
20 covalent internucleoside (backbone) linkages as well as
oligonucleotides having non-naturally-occurring portions
which function similarly. Such modified or substituted
oligonucleotides are often preferred over native forms
because of desirable properties such as, for example,
25 enhanced cellular uptake, enhanced affinity for nucleic
acid target and increased stability in the presence of
nucleases.

While antisense oligonucleotides are a preferred form of
antisense compound, the present invention comprehends other
30 oligomeric antisense compounds, including but not limited
to oligonucleotide mimetics such as are described below.
The antisense compounds in accordance with this invention
preferably comprise from about 8 to about 30 nucleobases
(i.e. from about 8 to about 30 linked nucleosides).
35 Particularly preferred antisense compounds are antisense

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oligonucleotides, even more preferably those comprising
from about 12 to about 25 nucleobases. As is known in the
art, a nucleoside is a base-sugar combination. The base
portion of the nucleoside is normally a heterocyclic base.
5   The two most common classes of such heterocyclic bases are
the purines and the pyrimidines. Nucleotides are
nucleosides that further include a phosphate group
covalently linked to the sugar portion of the nucleoside.
For those nucleosides that include a pentofuranosyl sugar,
10 the phosphate group can be linked to either the 2', 3' or
5' hydroxyl moiety of the sugar. In forming
oligonucleotides, the phosphate groups covalently link
adjacent nucleosides to one another to form a linear
polymeric compound. In turn the respective ends of this
15 linear polymeric structure can be further joined to form a
circular structure, however, open linear structures are
generally preferred. Within the oligonucleotide structure,
the phosphate groups are commonly referred to as forming
the internucleoside backbone of the oligonucleotide. The
20 normal linkage or backbone of RNA and DNA is a 3' to 5'
phosphodiester linkage.

    Specific examples of preferred antisense compounds
useful in this invention include oligonucleotides
containing modified backbones or non-natural
25 internucleoside linkages. As defined in this
specification, oligonucleotides having modified backbones
include those that retain a phosphorus atom in the backbone
and those that do not have a phosphorus atom in the
backbone. For the purposes of this specification, and as
30 sometimes referenced in the art, modified oligonucleotides
that do not have a phosphorus atom in their internucleoside
backbone can also be considered to be oligonucleosides.

    Preferred modified oligonucleotide backbones include,
for example, phosphorothioates, chiral phosphorothioates,
35 phosphorodithioates, phosphotriesters, aminoalkyl-

phosphotriesters, methyl and other alkyl phosphonates
including 3'-alkylene phosphonates and chiral phosphonates,
phosphinates, phosphoramidates including 3'-amino
phosphoramidate and aminoalkylphosphoramidates,
5 thionophosphoramidates, thionoalkylphosphonates,
thionoalkylphosphotriesters, and boranophosphates having
normal 3'-5' linkages, 2'-5' linked analogs of these, and
those having inverted polarity wherein the adjacent pairs
of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to
10 5'-2'. Various salts, mixed salts and free acid forms are
also included.

Representative United States patents that teach the
preparation of the above phosphorus-containing linkages
include, but are not limited to, U.S.: 3,687,808;
15 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897;
5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131;
5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677;
5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111;
5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of
20 which are commonly owned with this application, and each of
which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not
include a phosphorus atom therein have backbones that are
formed by short chain alkyl or cycloalkyl internucleoside
25 linkages, mixed heteroatom and alkyl or cycloalkyl
internucleoside linkages, or one or more short chain
heteroatomic or heterocyclic internucleoside linkages.
These include those having morpholino linkages (formed in
part from the sugar portion of a nucleoside); siloxane
30 backbones; sulfide, sulfoxide and sulfone backbones;
formacetyl and thioformacetyl backbones; methylene
formacetyl and thioformacetyl backbones; alkene containing
backbones; sulfamate backbones; methyleneimino and
methylenehydrazino backbones; sulfonate and sulfonamide

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backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are
5 not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070;
10 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone,
15 of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is
20 referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza
25 nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching
30 of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in
35 particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a

methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$,
$-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the
native phosphodiester backbone is represented as $-O-P-O-CH_2-$
] of the above referenced U.S. patent 5,489,677, and the
5 amide backbones of the above referenced U.S. patent
5,602,240. Also preferred are oligonucleotides having
morpholino backbone structures of the above-referenced U.S.
patent 5,034,506.

Modified oligonucleotides may also contain one or more
10 substituted sugar moieties. Preferred oligonucleotides
comprise one of the following at the 2' position: OH; F; O-
, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-
alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and
alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl
15 or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are
$O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$,
and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about
10. Other preferred oligonucleotides comprise one of the
following at the 2' position: C_1 to C_{10} lower alkyl,
20 substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-
aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3,
ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl,
aminoalkylamino, polyalkylamino, substituted silyl, an RNA
cleaving group, a reporter group, an intercalator, a group
25 for improving the pharmacokinetic properties of an
oligonucleotide, or a group for improving the
pharmacodynamic properties of an oligonucleotide, and other
substituents having similar properties. A preferred
modification includes 2'-methoxyethoxy ($2'-O-CH_2CH_2OCH_3$, also
30 known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al.,
Helv. Chim. Acta, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy
group. A further preferred modification includes 2'-
dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also
known as 2'-DMAOE, as described in examples hereinbelow,
35 and 2'-dimethylaminoethoxyethoxy (also known in the art as

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2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH$_2$-
O-CH$_2$-N(CH$_2$)$_2$, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-
CH$_3$), 2'-aminopropoxy (2'-OCH$_2$CH$_2$CH$_2$NH$_2$) and 2'-fluoro (2'-
5   F). Similar modifications may also be made at other
positions on the oligonucleotide, particularly the 3'
position of the sugar on the 3' terminal nucleotide or in
2'-5' linked oligonucleotides and the 5' position of 5'
terminal nucleotide. Oligonucleotides may also have sugar
10 mimetics such as cyclobutyl moieties in place of the
pentofuranosyl sugar. Representative United States patents
that teach the preparation of such modified sugar
structures include, but are not limited to, U.S.:
4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;
15 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;
5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053;
5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920,
certain of which are commonly owned with the instant
application, and each of which is herein incorporated by
20 reference in its entirety.

Oligonucleotides may also include nucleobase (often
referred to in the art simply as "base") modifications or
substitutions. As used herein, "unmodified" or "natural"
nucleobases include the purine bases adenine (A) and
25 guanine (G), and the pyrimidine bases thymine (T), cytosine
(C) and uracil (U). Modified nucleobases include other
synthetic and natural nucleobases such as 5-methylcytosine
(5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine,
2-aminoadenine, 6-methyl and other alkyl derivatives of
30 adenine and guanine, 2-propyl and other alkyl derivatives
of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-
thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil
and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil
(pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-
35 thioalkyl, 8-hydroxyl and other 8-substituted adenines and

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guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl
and other 5-substituted uracils and cytosines, 7-
methylguanine and 7-methyladenine, 8-azaguanine and 8-
azaadenine, 7-deazaguanine and 7-deazaadenine and 3-
5 deazaguanine and 3-deazaadenine. Further nucleobases
include those disclosed in United States Patent No.
3,687,808, those disclosed in *The Concise Encyclopedia Of
Polymer Science And Engineering*, pages 858-859, Kroschwitz,
J.I., ed. John Wiley & Sons, **1990**, those disclosed by
10 Englisch et al., *Angewandte Chemie*, International Edition,
1991, *30*, 613, and those disclosed by Sanghvi, Y.S.,
Chapter 15, *Antisense Research and Applications*, pages 289-
302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**.
Certain of these nucleobases are particularly useful for
15 increasing the binding affinity of the oligomeric compounds
of the invention. These include 5-substituted pyrimidines,
6-azapyrimidines and N-2, N-6 and O-6 substituted purines,
including 2-aminopropyladenine, 5-propynyluracil and 5-
propynylcytosine. 5-methylcytosine substitutions have been
20 shown to increase nucleic acid duplex stability by 0.6-1.2°C
(Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds.,
Antisense Research and Applications, CRC Press, Boca Raton,
1993, pp. 276-278) and are presently preferred base
substitutions, even more particularly when combined with
25 2'-O-methoxyethyl sugar modifications.

     Representative United States patents that teach the
preparation of certain of the above noted modified
nucleobases as well as other modified nucleobases include,
but are not limited to, the above noted U.S. 3,687,808, as
30 well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273;
5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908;
5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121,
5,596,091; 5,614,617; and 5,681,941, certain of which are
commonly owned with the instant application, and each of

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which is herein incorporated by reference, and United
States patent 5,750,692, which is commonly owned with the
instant application and also herein incorporated by
reference.

5   Another modification of the oligonucleotides of the
invention involves chemically linking to the
oligonucleotide one or more moieties or conjugates which
enhance the activity, cellular distribution or cellular
uptake of the oligonucleotide. Such moieties include but
10 are not limited to lipid moieties such as a cholesterol
moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**,
86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med.
Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-
S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*,
15 **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem.
Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et
al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic
chain, e.g., dodecandiol or undecyl residues (Saison-
Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et
20 al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al.,
Biochimie, **1993**, *75*, 49-54), a phospholipid, e.g., di-
hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-
hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al.,
Tetrahedron Lett., **1995**, *36*, 3651-3654; Shea et al., *Nucl.
25 Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a
polyethylene glycol chain (Manoharan et al., *Nucleosides &
Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid
(Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654),
a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*,
30 **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-
carbonyl-oxycholesterol moiety (Crooke et al., *J.
Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937.

Representative United States patents that teach the
preparation of such oligonucleotide conjugates include, but

are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105;
5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717,
5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802;
5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718;
5   5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779;
4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582;
4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;
5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506;
5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,
10 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667;
5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481;
5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and
5,688,941, certain of which are commonly owned with the
instant application, and each of which is herein
15 incorporated by reference.

    It is not necessary for all positions in a given
compound to be uniformly modified, and in fact more than
one of the aforementioned modifications may be incorporated
in a single compound or even at a single nucleoside within
20 an oligonucleotide. The present invention also includes
antisense compounds which are chimeric compounds.
"Chimeric" antisense compounds or "chimeras," in the
context of this invention, are antisense compounds,
particularly oligonucleotides, which contain two or more
25 chemically distinct regions, each made up of at least one
monomer unit, i.e., a nucleotide in the case of an
oligonucleotide compound. These oligonucleotides typically
contain at least one region wherein the oligonucleotide is
modified so as to confer upon the oligonucleotide increased
30 resistance to nuclease degradation, increased cellular
uptake, and/or increased binding affinity for the target
nucleic acid. An additional region of the oligonucleotide
may serve as a substrate for enzymes capable of cleaving
RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is
35 a cellular endonuclease which cleaves the RNA strand of an

RNA:DNA duplex. Activation of RNase H, therefore, results
in cleavage of the RNA target, thereby greatly enhancing
the efficiency of oligonucleotide inhibition of gene
expression. Consequently, comparable results can often be
5   obtained with shorter oligonucleotides when chimeric
oligonucleotides are used, compared to phosphorothioate
deoxyoligonucleotides hybridizing to the same target
region. Cleavage of the RNA target can be routinely
detected by gel electrophoresis and, if necessary,
10 associated nucleic acid hybridization techniques known in
the art.

     Chimeric antisense compounds of the invention may be
formed as composite structures of two or more
oligonucleotides, modified oligonucleotides,
15 oligonucleosides and/or oligonucleotide mimetics as
described above. Such compounds have also been referred to
in the art as hybrids or gapmers. Representative United
States patents that teach the preparation of such hybrid
structures include, but are not limited to, U.S.:
20 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878;
5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355;
5,652,356; and 5,700,922, certain of which are commonly
owned with the instant application, and each of which is
herein incorporated by reference in its entirety.

25   The antisense compounds used in accordance with this
invention may be conveniently and routinely made through
the well-known technique of solid phase synthesis.
Equipment for such synthesis is sold by several vendors
including, for example, Applied Biosystems (Foster City,
30 CA). Any other means for such synthesis known in the art
may additionally or alternatively be employed. It is well
known to use similar techniques to prepare oligonucleotides
such as the phosphorothioates and alkylated derivatives.
     The antisense compounds of the invention are synthesized
35 in vitro and do not include antisense compositions of

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biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules. The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other

5 molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such

10 uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.:
5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;
5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;

15 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;
5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;
5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any

20 pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the

25 disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is

30 prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE

35 [(S-acetyl-2-thioethyl) phosphate] derivatives according to

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the methods disclosed in WO 93/24510 to Gosselin et al.,
published December 9, 1993 or in WO 94/26764 to Imbach et
al.

        The term "pharmaceutically acceptable salts" refers to
5   physiologically and pharmaceutically acceptable salts of
    the compounds of the invention: i.e., salts that retain the
    desired biological activity of the parent compound and do
    not impart undesired toxicological effects thereto.

        Pharmaceutically acceptable base addition salts are
10 formed with metals or amines, such as alkali and alkaline
    earth metals or organic amines. Examples of metals used as
    cations are sodium, potassium, magnesium, calcium, and the
    like. Examples of suitable amines are
    N,N'-dibenzylethylenediamine, chloroprocaine, choline,
15 diethanolamine, dicyclohexylamine, ethylenediamine,
    N-methylglucamine, and procaine (see, for example, Berge et
    al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*,
    1-19). The base addition salts of said acidic compounds
    are prepared by contacting the free acid form with a
20 sufficient amount of the desired base to produce the salt
    in the conventional manner. The free acid form may be
    regenerated by contacting the salt form with an acid and
    isolating the free acid in the conventional manner. The
    free acid forms differ from their respective salt forms
25 somewhat in certain physical properties such as solubility
    in polar solvents, but otherwise the salts are equivalent
    to their respective free acid for purposes of the present
    invention. As used herein, a "pharmaceutical addition
    salt" includes a pharmaceutically acceptable salt of an
30 acid form of one of the components of the compositions of
    the invention. These include organic or inorganic acid
    salts of the amines. Preferred acid salts are the
    hydrochlorides, acetates, salicylates, nitrates and
    phosphates. Other suitable pharmaceutically acceptable
35 salts are well known to those skilled in the art and

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include basic salts of a variety of inorganic and organic
acids, such as, for example, with inorganic acids, such as
for example hydrochloric acid, hydrobromic acid, sulfuric
acid or phosphoric acid; with organic carboxylic, sulfonic,
5 sulfo or phospho acids or N-substituted sulfamic acids, for
example acetic acid, propionic acid, glycolic acid,
succinic acid, maleic acid, hydroxymaleic acid,
methylmaleic acid, fumaric acid, malic acid, tartaric acid,
lactic acid, oxalic acid, gluconic acid, glucaric acid,
10 glucuronic acid, citric acid, benzoic acid, cinnamic acid,
mandelic acid, salicylic acid, 4-aminosalicylic acid,
2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid,
nicotinic acid or isonicotinic acid; and with amino acids,
such as the 20 alpha-amino acids involved in the synthesis
15 of proteins in nature, for example glutamic acid or
aspartic acid, and also with phenylacetic acid,
methanesulfonic acid, ethanesulfonic acid,
2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid,
benzenesulfonic acid, 4-methylbenzenesulfoic acid,
20 naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic
acid, 2- or 3-phosphoglycerate, glucose-6-phosphate,
N-cyclohexylsulfamic acid (with the formation of
cyclamates), or with other acid organic compounds, such as
ascorbic acid. Pharmaceutically acceptable salts of
25 compounds may also be prepared with a pharmaceutically
acceptable cation. Suitable pharmaceutically acceptable
cations are well known to those skilled in the art and
include alkaline, alkaline earth, ammonium and quaternary
ammonium cations. Carbonates or hydrogen carbonates are
30 also possible.
        For oligonucleotides, preferred examples of
pharmaceutically acceptable salts include but are not
limited to (a) salts formed with cations such as sodium,
potassium, ammonium, magnesium, calcium, polyamines such as
35 spermine and spermidine, etc.; (b) acid addition salts

formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric

5 acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid,

10 polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as

15 research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of liver glycogen phosphorylase is treated by administering antisense compounds in accordance with this invention. The

20 compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful

25 prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding liver glycogen phosphorylase,

30 enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding liver glycogen phosphorylase can be detected by means known in the art. Such means may include conjugation

35 of an enzyme to the oligonucleotide, radiolabelling of the

oligonucleotide or any other suitable detection means.
Kits using such detection means for detecting the level of
liver glycogen phosphorylase in a sample may also be
prepared.

5   The present invention also includes pharmaceutical
compositions and formulations which include the antisense
compounds of the invention. The pharmaceutical
compositions of the present invention may be administered
in a number of ways depending upon whether local or

10 systemic treatment is desired and upon the area to be
treated. Administration may be topical (including
ophthalmic and to mucous membranes including vaginal and
rectal delivery), pulmonary, e.g., by inhalation or
insufflation of powders or aerosols, including by

15 nebulizer; intratracheal, intranasal, epidermal and
transdermal), oral or parenteral. Parenteral
administration includes intravenous, intraarterial,
subcutaneous, intraperitoneal or intramuscular injection or
infusion; or intracranial, e.g., intrathecal or

20 intraventricular, administration. Oligonucleotides with at
least one 2'-O-methoxyethyl modification are believed to be
particularly useful for oral administration.

        Pharmaceutical compositions and formulations for topical
administration may include transdermal patches, ointments,

25 lotions, creams, gels, drops, suppositories, sprays,
liquids and powders. Conventional pharmaceutical carriers,
aqueous, powder or oily bases, thickeners and the like may
be necessary or desirable. Coated condoms, gloves and the
like may also be useful.

30   Compositions and formulations for oral administration
include powders or granules, suspensions or solutions in
water or non-aqueous media, capsules, sachets or tablets.
Thickeners, flavoring agents, diluents, emulsifiers,
dispersing aids or binders may be desirable.

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Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not
5 limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may
10 be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit
15 dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared
20 by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be
25 formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions
30 may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the
35 pharmaceutical compositions may be formulated and used as

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foams. Pharmaceutical foams include formulations such as,
but not limited to, emulsions, microemulsions, creams,
jellies and liposomes. While basically similar in nature
these formulations vary in the components and the
5 consistency of the final product. The preparation of such
compositions and formulations is generally known to those
skilled in the pharmaceutical and formulation arts and may
be applied to the formulation of the compositions of the
present invention.
10

Emulsions
      The compositions of the present invention may be
prepared and formulated as emulsions. Emulsions are
typically heterogenous systems of one liquid dispersed in
15 another in the form of droplets usually exceeding 0.1 μm in
diameter. (Idson, in *Pharmaceutical Dosage Forms*,
Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker,
Inc., New York, N.Y., volume 1, p. 199; Rosoff, in
Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker
20 (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., Volume
1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman,
Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New
York, N.Y., volume 2, p. 335; Higuchi *et al.*, in
Remington's Pharmaceutical Sciences, Mack Publishing Co.,
25 Easton, PA, **1985**, p. 301). Emulsions are often biphasic
systems comprising of two immiscible liquid phases
intimately mixed and dispersed with each other. In
general, emulsions may be either water-in-oil (w/o) or of
the oil-in-water (o/w) variety. When an aqueous phase is
30 finely divided into and dispersed as minute droplets into a
bulk oily phase the resulting composition is called a
water-in-oil (w/o) emulsion. Alternatively, when an oily
phase is finely divided into and dispersed as minute
droplets into a bulk aqueous phase the resulting

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composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily

5   phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for

10 example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small

15 water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no

20 thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may

25 be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories:

30 synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

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Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and
5   Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a
10 hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified
15 into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).
20   Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as
25 anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as
30 bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.
    A large variety of non-emulsifying materials are also
35 included in emulsion formulations and contribute to the

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properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger

5   and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally

10 occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for

15 example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external

20 phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly

25 used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the

30 formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid,

35 and lecithin.

The application of emulsion formulations via
dermatological, oral and parenteral routes and methods for
their manufacture have been reviewed in the literature
(Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger

5   and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York,
N.Y., volume 1, p. 199). Emulsion formulations for oral
delivery have been very widely used because of reasons of
ease of formulation, efficacy from an absorption and
bioavailability standpoint. (Rosoff, in *Pharmaceutical*

10 *Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**,
Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245;
Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York,
N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-

15 soluble vitamins and high fat nutritive preparations are
among the materials that have commonly been administered
orally as o/w emulsions.

In one embodiment of the present invention, the
compositions of oligonucleotides and nucleic acids are

20 formulated as microemulsions. A microemulsion may be
defined as a system of water, oil and amphiphile which is a
single optically isotropic and thermodynamically stable
liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*,
Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker,

25 Inc., New York, N.Y., volume 1, p. 245). Typically
microemulsions are systems that are prepared by first
dispersing an oil in an aqueous surfactant solution and
then adding a sufficient amount of a fourth component,
generally an intermediate chain-length alcohol to form a

30 transparent system. Therefore, microemulsions have also
been described as thermodynamically stable, isotropically
clear dispersions of two immiscible liquids that are
stabilized by interfacial films of surface-active molecules
(Leung and Shah, *in: Controlled Release of Drugs: Polymers*

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and *Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH
Publishers, New York, pages 185-215). Microemulsions
commonly are prepared via a combination of three to five
components that include oil, water, surfactant,

5 cosurfactant and electrolyte. Whether the microemulsion is
of the water-in-oil (w/o) or an oil-in-water (o/w) type is
dependent on the properties of the oil and surfactant used
and on the structure and geometric packing of the polar
heads and hydrocarbon tails of the surfactant molecules

10 (Schott, in *Remington's Pharmaceutical Sciences*, Mack
Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams
has been extensively studied and has yielded a
comprehensive knowledge, to one skilled in the art, of how

15 to formulate microemulsions (Rosoff, in *Pharmaceutical
Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**,
Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245;
Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York,

20 N.Y., volume 1, p. 335). Compared to conventional
emulsions, microemulsions offer the advantage of
solubilizing water-insoluble drugs in a formulation of
thermodynamically stable droplets that are formed
spontaneously.

25 Surfactants used in the preparation of microemulsions
include, but are not limited to, ionic surfactants, non-
ionic surfactants, Brij 96, polyoxyethylene oleyl ethers,
polyglycerol fatty acid esters, tetraglycerol monolaurate
(ML310), tetraglycerol monooleate (MO310), hexaglycerol

30 monooleate (PO310), hexaglycerol pentaoleate (PO500),
decaglycerol monocaprate (MCA750), decaglycerol monooleate
(MO750), decaglycerol sequioleate (SO750), decaglycerol
decaoleate (DAO750), alone or in combination with
cosurfactants. The cosurfactant, usually a short-chain

alcohol such as ethanol, 1-propanol, and 1-butanol, serves
to increase the interfacial fluidity by penetrating into
the surfactant film and consequently creating a disordered
film because of the void space generated among surfactant
5   molecules. Microemulsions may, however, be prepared
without the use of cosurfactants and alcohol-free self-
emulsifying microemulsion systems are known in the art.
The aqueous phase may typically be, but is not limited to,
water, an aqueous solution of the drug, glycerol, PEG300,
10 PEG400, polyglycerols, propylene glycols, and derivatives
of ethylene glycol. The oil phase may include, but is not
limited to, materials such as Captex 300, Captex 355,
Capmul MCM, fatty acid esters, medium chain (C8-C12) mono,
di, and tri-glycerides, polyoxyethylated glyceryl fatty
15 acid esters, fatty alcohols, polyglycolized glycerides,
saturated polyglycolized C8-C10 glycerides, vegetable oils
and silicone oil.

Microemulsions are particularly of interest from the
standpoint of drug solubilization and the enhanced
20 absorption of drugs. Lipid based microemulsions (both o/w
and w/o) have been proposed to enhance the oral
bioavailability of drugs, including peptides
(Constantinides et al., *Pharmaceutical Research*, **1994**, *11*,
1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*,
25 **1993**, *13*, 205). Microemulsions afford advantages of
improved drug solubilization, protection of drug from
enzymatic hydrolysis, possible enhancement of drug
absorption due to surfactant-induced alterations in
membrane fluidity and permeability, ease of preparation,
30 ease of oral administration over solid dosage forms,
improved clinical potency, and decreased toxicity
(Constantinides et al., *Pharmaceutical Research*, **1994**, *11*,
1385; Ho et al., *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often
microemulsions may form spontaneously when their components

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are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active

5 components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the

10 gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain

15 additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the

20 microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al., Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p. 92). Each of

25 these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the

30 formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term

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"liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and
5   an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages
10 *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a
15 liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of
20 water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245).
25 Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the
30 liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the
35 active agent may act.

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Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other

5 formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into

10 the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been

15 administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable

20 complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res.*

25 *Commun.*, **1987**, *147*, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is

30 entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled*

35 *Release*, **1992**, *19*, 269-274).

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One major type of liposomal composition includes
phospholipids other than naturally-derived
phosphatidylcholine. Neutral liposome compositions, for
example, can be formed from dimyristoyl phosphatidylcholine
5   (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic
liposome compositions generally are formed from dimyristoyl
phosphatidylglycerol, while anionic fusogenic liposomes are
formed primarily from dioleoyl phosphatidylethanolamine
(DOPE). Another type of liposomal composition is formed
10   from phosphatidylcholine (PC) such as, for example, soybean
PC, and egg PC. Another type is formed from mixtures of
phospholipid and/or phosphatidylcholine and/or cholesterol.
    Several studies have assessed the topical delivery of
liposomal drug formulations to the skin. Application of
15   liposomes containing interferon to guinea pig skin resulted
in a reduction of skin herpes sores while delivery of
interferon via other means (e.g. as a solution or as an
emulsion) were ineffective (Weiner et al., Journal of Drug
Targeting, 1992, 2, 405-410). Further, an additional study
20   tested the efficacy of interferon administered as part of a
liposomal formulation to the administration of interferon
using an aqueous system, and concluded that the liposomal
formulation was superior to aqueous administration (du
Plessis et al., Antiviral Research, 1992, 18, 259-265).
25   Non-ionic liposomal systems have also been examined to
determine their utility in the delivery of drugs to the
skin, in particular systems comprising non-ionic surfactant
and cholesterol. Non-ionic liposomal formulations
comprising Novasome™ I (glyceryl
30   dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and
Novasome™ II (glyceryl distearate/
cholesterol/polyoxyethylene-10-stearyl ether) were used to
deliver cyclosporin-A into the dermis of mouse skin.
Results indicated that such non-ionic liposomal systems
35   were effective in facilitating the deposition of

cyclosporin-A into different layers of the skin (Hu et al. *S.T.P.Pharma. Sci.*, **1994**, 4, 6, 466).

Liposomes also include "sterically stabilized"
liposomes, a term which, as used herein, refers to
5 liposomes comprising one or more specialized lipids that,
when incorporated into liposomes, result in enhanced
circulation lifetimes relative to liposomes lacking such
specialized lipids. Examples of sterically stabilized
liposomes are those in which part of the vesicle-forming
10 lipid portion of the liposome (A) comprises one or more
glycolipids, such as monosialoganglioside G_{M1}, or (B) is
derivatized with one or more hydrophilic polymers, such as
a polyethylene glycol (PEG) moiety. While not wishing to
be bound by any particular theory, it is thought in the art
15 that, at least for sterically stabilized liposomes
containing gangliosides, sphingomyelin, or PEG-derivatized
lipids, the enhanced circulation half-life of these
sterically stabilized liposomes derives from a reduced
uptake into cells of the reticuloendothelial system (RES)
20 (Allen *et al.*, *FEBS Letters*, **1987,** 223, 42; Wu *et al.*,
Cancer Research, **1993,** 53, 3765).   Various
liposomes comprising one or more glycolipids are known in
the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*,
1987, 507, 64) reported the ability of
25 monosialoganglioside G_{M1}, galactocerebroside sulfate and
phosphatidylinositol to improve blood half-lives of
liposomes. These findings were expounded upon by Gabizon
et al. (*Proc. Natl. Acad. Sci. U.S.A.*, **1988,** 85, 6949).
U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen *et*
30 *al.*, disclose liposomes comprising (1) sphingomyelin and
(2) the ganglioside G_{M1} or a galactocerebroside sulfate
ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses
liposomes comprising sphingomyelin. Liposomes comprising

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1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in WO
97/13499 (Lim *et al.*).

Many liposomes comprising lipids derivatized with one or
more hydrophilic polymers, and methods of preparation

5 thereof, are known in the art. Sunamoto *et al.* (*Bull.
Chem. Soc. Jpn.*, **1980**, *53*, 2778) described liposomes
comprising a nonionic detergent, $2C_{12}15G$, that contains a
PEG moiety. Illum *et al.* (*FEBS Lett.*, **1984**, *167*, 79) noted
that hydrophilic coating of polystyrene particles with

10 polymeric glycols results in significantly enhanced blood
half-lives. Synthetic phospholipids modified by the
attachment of carboxylic groups of polyalkylene glycols
(*e.g.*, PEG) are described by Sears (U.S. Patent Nos.
4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Lett.*,

15 **1990**, *268*, 235) described experiments demonstrating that
liposomes comprising phosphatidylethanolamine (PE)
derivatized with PEG or PEG stearate have significant
increases in blood circulation half-lives. Blume *et al.*
(*Biochimica et Biophysica Acta,* **1990**, *1029*, 91) extended

20 such observations to other PEG-derivatized phospholipids,
e.g., DSPE-PEG, formed from the combination of
distearoylphosphatidylethanolamine (DSPE) and PEG.
Liposomes having covalently bound PEG moieties on their
external surface are described in European Patent No. EP 0

25 445 131 B1 and WO 90/04384 to Fisher. Liposome
compositions containing 1-20 mole percent of PE derivatized
with PEG, and methods of use thereof, are described by
Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633)
and Martin *et al.* (U.S. Patent No. 5,213,804 and European

30 Patent No. EP 0 496 813 B1). Liposomes comprising a number
of other lipid-polymer conjugates are disclosed in WO
91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et
al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes
comprising PEG-modified ceramide lipids are described in WO

96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935
(Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe
PEG-containing liposomes that can be further derivatized
with functional moieties on their surfaces.

5   A limited number of liposomes comprising nucleic acids
are known in the art. WO 96/40062 to Thierry *et al.*
discloses methods for encapsulating high molecular weight
nucleic acids in liposomes. U.S. Patent No. 5,264,221 to
Tagawa *et al.* discloses protein-bonded liposomes and

10 asserts that the contents of such liposomes may include an
antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.*
describes certain methods of encapsulating
oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et
al.* discloses liposomes comprising antisense

15 oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are
highly deformable lipid aggregates which are attractive
candidates for drug delivery vehicles. Transfersomes may
be described as lipid droplets which are so highly

20 deformable that they are easily able to penetrate through
pores which are smaller than the droplet. Transfersomes
are adaptable to the environment in which they are used,
e.g. they are self-optimizing (adaptive to the shape of
pores in the skin), self-repairing, frequently reach their

25 targets without fragmenting, and often self-loading. To
make transfersomes it is possible to add surface edge-
activators, usually surfactants, to a standard liposomal
composition. Transfersomes have been used to deliver serum
albumin to the skin. The transfersome-mediated delivery of

30 serum albumin has been shown to be as effective as
subcutaneous injection of a solution containing serum
albumin.

Surfactants find wide application in formulations such
as emulsions (including microemulsions) and liposomes. The

35 most common way of classifying and ranking the properties

of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for

5 categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants

10 find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol

15 esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The

20 polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include

25 carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important

30 members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include

35 quaternary ammonium salts and ethoxylated amines. The

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quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is
5 classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in
10 *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

Penetration Enhancers

In one embodiment, the present invention employs various
15 penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes.
20 It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the
25 permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic*
30 *Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention,
35 surfactants (or "surface-active agents") are chemical

entities which, when dissolved in an aqueous solution,
reduce the surface tension of the solution or the
interfacial tension between the aqueous solution and
another liquid, with the result that absorption of
5 oligonucleotides through the mucosa is enhanced. In
addition to bile salts and fatty acids, these penetration
enhancers include, for example, sodium lauryl sulfate,
polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl
ether) (Lee et al., *Critical Reviews in Therapeutic Drug*
10 *Carrier Systems*, **1991**, p.92); and perfluorochemical
emulsions, such as FC-43. Takahashi et al., *J. Pharm.
Pharmacol.*, **1988**, *40*, 252).

   Fatty acids: Various fatty acids and their derivatives
which act as penetration enhancers include, for example,
15 oleic acid, lauric acid, capric acid (n-decanoic acid),
myristic acid, palmitic acid, stearic acid, linoleic acid,
linolenic acid, dicaprate, tricaprate, monoolein (1-
monooleoyl-*rac*-glycerol), dilaurin, caprylic acid,
arachidonic acid, glycerol 1-monocaprate, 1-
20 dodecylazacycloheptan-2-one, acylcarnitines, acylcholines,
C_{1-10} alkyl esters thereof (e.g., methyl, isopropyl and *t*-
butyl), and mono- and di-glycerides thereof (i.e., oleate,
laurate, caprate, myristate, palmitate, stearate,
linoleate, etc.) (Lee et al., *Critical Reviews in
25 Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi,
Critical Reviews in Therapeutic Drug Carrier Systems, **1990**,
7, 1-33; El Hariri et al., J. Pharm. Pharmacol., **1992**, *44*,
651-654).

30   Bile salts: The physiological role of bile includes the
facilitation of dispersion and absorption of lipids and
fat-soluble vitamins (Brunton, Chapter 38 in: Goodman &
Gilman's *The Pharmacological Basis of Therapeutics*, 9th
Ed., Hardman et al. Eds., McGraw-Hill, New York, **1996**, pp.

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934-935). Various natural bile salts, and their synthetic
derivatives, act as penetration enhancers. Thus the term
"bile salts" includes any of the naturally occurring
components of bile as well as any of their synthetic

5 derivatives.   The bile salts of the invention include, for
example, cholic acid (or its pharmaceutically acceptable
sodium salt, sodium cholate), dehydrocholic acid (sodium
dehydrocholate), deoxycholic acid (sodium deoxycholate),
glucholic acid (sodium glucholate), glycholic acid (sodium

10 glycocholate), glycodeoxycholic acid (sodium
glycodeoxycholate), taurocholic acid (sodium taurocholate),
taurodeoxycholic acid (sodium taurodeoxycholate),
chenodeoxycholic acid (sodium chenodeoxycholate),
ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-

15 fusidate (STDHF), sodium glycodihydrofusidate and
polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical
Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92;
Swinyard, Chapter 39 *In: Remington's Pharmaceutical
Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co.,

20 Easton, PA, **1990**, pages 782-783; Muranishi, *Critical
Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33;
Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, **1992**, *263*, 25;
Yamashita *et al.*, *J. Pharm. Sci.*, **1990**, *79*, 579-583).

25   Chelating Agents: Chelating agents, as used in
connection with the present invention, can be defined as
compounds that remove metallic ions from solution by
forming complexes therewith, with the result that
absorption of oligonucleotides through the mucosa is

30 enhanced. With regards to their use as penetration
enhancers in the present invention, chelating agents have
the added advantage of also serving as DNase inhibitors, as
most characterized DNA nucleases require a divalent metal
ion for catalysis and are thus inhibited by chelating

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agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339).
Chelating agents of the invention include but are not
limited to disodium ethylenediaminetetraacetate (EDTA),
citric acid, salicylates (*e.g.*, sodium salicylate, 5-
5   methoxysalicylate and homovanilate), *N*-acyl derivatives of
collagen, laureth-9 and *N*-amino acyl derivatives of beta-
diketones (enamines)(Lee *et al.*, *Critical Reviews in
Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi,
Critical Reviews in Therapeutic Drug Carrier Systems, **1990**,
10   *7*, 1-33; Buur *et al.*, *J. Control Rel.*, **1990**, *14*, 43-51).

       Non-chelating non-surfactants: As used herein, non-
chelating non-surfactant penetration enhancing compounds
can be defined as compounds that demonstrate insignificant
15   activity as chelating agents or as surfactants but that
nonetheless enhance absorption of oligonucleotides through
the alimentary mucosa (Muranishi, *Critical Reviews in
Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33). This
class of penetration enhancers include, for example,
20   unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-
alkanone derivatives (Lee *et al.*, *Critical Reviews in
Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-
steroidal anti-inflammatory agents such as diclofenac
sodium, indomethacin and phenylbutazone (Yamashita *et al.*,
25   *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).
       Agents that enhance uptake of oligonucleotides at the
cellular level may also be added to the pharmaceutical and
other compositions of the present invention. For example,
cationic lipids, such as lipofectin (Junichi *et al*, U.S.
30   Patent No. 5,705,188), cationic glycerol derivatives, and
polycationic molecules, such as polylysine (Lollo *et al.*,
PCT Application WO 97/30731), are also known to enhance the
cellular uptake of oligonucleotides.

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Other agents may be utilized to enhance the penetration
of the administered nucleic acids, including glycols such
as ethylene glycol and propylene glycol, pyrrols such as 2-
pyrrol, azones, and terpenes such as limonene and menthone.

5 Carriers

Certain compositions of the present invention also
incorporate carrier compounds in the formulation. As used
herein, "carrier compound" or "carrier" can refer to a
nucleic acid, or analog thereof, which is inert (*i.e.*, does

10 not possess biological activity *per se*) but is recognized
as a nucleic acid by *in vivo* processes that reduce the
bioavailability of a nucleic acid having biological
activity by, for example, degrading the biologically active
nucleic acid or promoting its removal from circulation.

15 The coadministration of a nucleic acid and a carrier
compound, typically with an excess of the latter substance,
can result in a substantial reduction of the amount of
nucleic acid recovered in the liver, kidney or other
extracirculatory reservoirs, presumably due to competition

20 between the carrier compound and the nucleic acid for a
common receptor. For example, the recovery of a partially
phosphorothioate oligonucleotide in hepatic tissue can be
reduced when it is coadministered with polyinosinic acid,
dextran sulfate, polycytidic acid or 4-acetamido-

25 4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*,
Antisense Res. Dev., **1995**, *5*, 115-121; Takakura *et al.*,
Antisense & Nucl. Acid Drug Dev., **1996**, *6*, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical

30 carrier" or "excipient" is a pharmaceutically acceptable
solvent, suspending agent or any other pharmacologically
inert vehicle for delivering one or more nucleic acids to
an animal. The excipient may be liquid or solid and is
selected, with the planned manner of administration in

35 mind, so as to provide for the desired bulk, consistency,

etc., when combined with a nucleic acid and the other
components of a given pharmaceutical composition. Typical
pharmaceutical carriers include, but are not limited to,
binding agents (*e.g.*, pregelatinized maize starch,
5   polyvinylpyrrolidone or hydroxypropyl methylcellulose,
etc.); fillers (*e.g.*, lactose and other sugars,
microcrystalline cellulose, pectin, gelatin, calcium
sulfate, ethyl cellulose, polyacrylates or calcium hydrogen
phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate,
10 talc, silica, colloidal silicon dioxide, stearic acid,
metallic stearates, hydrogenated vegetable oils, corn
starch, polyethylene glycols, sodium benzoate, sodium
acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch
glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl
15 sulphate, *etc.*).

   Pharmaceutically acceptable organic or inorganic
excipient suitable for non-parenteral administration which
do not deleteriously react with nucleic acids can also be
used to formulate the compositions of the present
20 invention. Suitable pharmaceutically acceptable carriers
include, but are not limited to, water, salt solutions,
alcohols, polyethylene glycols, gelatin, lactose, amylose,
magnesium stearate, talc, silicic acid, viscous paraffin,
hydroxymethylcellulose, polyvinylpyrrolidone and the like.
25   Formulations for topical administration of nucleic acids
may include sterile and non-sterile aqueous solutions, non-
aqueous solutions in common solvents such as alcohols, or
solutions of the nucleic acids in liquid or solid oil
bases. The solutions may also contain buffers, diluents and
30 other suitable additives. Pharmaceutically acceptable
organic or inorganic excipients suitable for non-parenteral
administration which do not deleteriously react with
nucleic acids can be used.

   Suitable pharmaceutically acceptable excipients include,
35 but are not limited to, water, salt solutions, alcohol,

polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

5   The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible,

10 pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as

15 dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations

20 can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact

25 with the nucleic acid(s) of the formulation.

      Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

30   Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents

35 include, but are not limited to, anticancer drugs such as

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daunorubicin, dactinomycin, doxorubicin, bleomycin,
mitomycin, nitrogen mustard, chlorambucil, melphalan,
cyclophosphamide, 6-mercaptopurine, 6-thioguanine,
cytarabine (CA), 5-fluorouracil (5-FU), floxuridine

5   (5-FUdR), methotrexate (MTX), colchicine, vincristine,
vinblastine, etoposide, teniposide, cisplatin and
diethylstilbestrol (DES). See, generally, *The Merck Manual
of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds.,
1987, Rahway, N.J., pages 1206-1228). Anti-inflammatory

10 drugs, including but not limited to nonsteroidal anti-
inflammatory drugs and corticosteroids, and antiviral
drugs, including but not limited to ribivirin, vidarabine,
acyclovir and ganciclovir, may also be combined in
compositions of the invention. See, generally, *The Merck*

15 *Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al.,
eds., **1987**, Rahway, N.J., pages 2499-2506 and 46-49,
respectively). Other non-antisense chemotherapeutic agents
are also within the scope of this invention. Two or more
combined compounds may be used together or sequentially.

20   In another related embodiment, compositions of the
invention may contain one or more antisense compounds,
particularly oligonucleotides, targeted to a first nucleic
acid and one or more additional antisense compounds
targeted to a second nucleic acid target. Numerous examples

25 of antisense compounds are known in the art. Two or more
combined compounds may be used together or sequentially.

        The formulation of therapeutic compositions and their
subsequent administration is believed to be within the
skill of those in the art. Dosing is dependent on severity

30 and responsiveness of the disease state to be treated, with
the course of treatment lasting from several days to
several months, or until a cure is effected or a diminution
of the disease state is achieved. Optimal dosing schedules
can be calculated from measurements of drug accumulation in

35 the body of the patient. Persons of ordinary skill can

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easily determine optimum dosages, dosing methodologies and
repetition rates. Optimum dosages may vary depending on
the relative potency of individual oligonucleotides, and
can generally be estimated based on EC_{50}s found to be
5 effective in in vitro and in vivo animal models. In
general, dosage is from 0.01 ug to 100 g per kg of body
weight, and may be given once or more daily, weekly,
monthly or yearly, or even once every 2 to 20 years.
Persons of ordinary skill in the art can easily estimate
10 repetition rates for dosing based on measured residence
times and concentrations of the drug in bodily fluids or
tissues. Following successful treatment, it may be
desirable to have the patient undergo maintenance therapy
to prevent the recurrence of the disease state, wherein the
15 oligonucleotide is administered in maintenance doses,
ranging from 0.01 ug to 100 g per kg of body weight, once
or more daily, to once every 20 years.

        While the present invention has been described with
specificity in accordance with certain of its preferred
20 embodiments, the following examples serve only to
illustrate the invention and are not intended to limit the
same.

EXAMPLES

Example 1

25 **Nucleoside Phosphoramidites for Oligonucleotide Synthesis**
Deoxy and 2'-alkoxy amidites

        2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl
phosphoramidites were purchased from commercial sources
(e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling
30 VA). Other 2'-O-alkoxy substituted nucleoside amidites are
prepared as described in U.S. Patent 5,506,351, herein
incorporated by reference. For oligonucleotides
synthesized using 2'-alkoxy amidites, the standard cycle
for unmodified oligonucleotides was utilized, except the

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wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to

5   published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

10   **2'-Fluorodeoxyadenosine amidites**

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, *36*, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected

15   nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl

20   group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to

25   obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS)

30   protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected

arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and
5   5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was
10   treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via
15   amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

20   2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

25   5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved
30   carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the

residue was dissolved in a minimum amount of methanol (ca.
400 mL). The solution was poured into fresh ether (2.5 L)
to yield a stiff gum. The ether was decanted and the gum
was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to
5   give a solid that was crushed to a light tan powder (57 g,
85% crude yield). The NMR spectrum was consistent with the
structure, contaminated with phenol as its sodium salt (ca.
5%). The material was used as is for further reactions (or
it can be purified further by column chromatography using a
10 gradient of methanol in ethyl acetate (10-25%) to give a
white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-
methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol
15 (1.2 L) were added to a 2 L stainless steel pressure vessel
and placed in a pre-heated oil bath at 160°C. After heating
for 48 hours at 155-160°C, the vessel was opened and the
solution evaporated to dryness and triturated with MeOH
(200 mL). The residue was suspended in hot acetone (1 L).
20 The insoluble salts were filtered, washed with acetone (150
mL) and the filtrate evaporated. The residue (280 g) was
dissolved in CH_3CN (600 mL) and evaporated. A silica gel
column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3)
containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2
25 (250 mL) and adsorbed onto silica (150 g) prior to loading
onto the column. The product was eluted with the packing
solvent to give 160 g (63%) of product. Additional
material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

30 2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was
co-evaporated with pyridine (250 mL) and the dried residue
dissolved in pyridine (1.3 L). A first aliquot of
dimethoxytrityl chloride (94.3 g, 0.278 M) was added and
the mixture stirred at room temperature for one hour. A
35 second aliquot of dimethoxytrityl chloride (94.3 g, 0.278

M) was added and the reaction stirred for an additional one
hour. Methanol (170 mL) was then added to stop the
reaction. HPLC showed the presence of approximately 70%
product. The solvent was evaporated and triturated with
5   CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L)
and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL
of saturated NaCl. The organic phase was dried over Na₂SO₄,
filtered and evaporated. 275 g of residue was obtained.
The residue was purified on a 3.5 kg silica gel column,
10 packed and eluted with EtOAc/hexane/acetone (5:5:1)
containing 0.5% Et₃NH. The pure fractions were evaporated
to give 164 g of product. Approximately 20 g additional
was obtained from the impure fractions to give a total
yield of 183 g (57%).

15   **3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-**
methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine
(106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture
prepared from 562 mL of DMF and 188 mL of pyridine) and
20 acetic anhydride (24.38 mL, 0.258 M) were combined and
stirred at room temperature for 24 hours. The reaction was
monitored by TLC by first quenching the TLC sample with the
addition of MeOH. Upon completion of the reaction, as
judged by TLC, MeOH (50 mL) was added and the mixture
25 evaporated at 35°C. The residue was dissolved in CHCl₃ (800
mL) and extracted with 2x200 mL of saturated sodium
bicarbonate and 2x200 mL of saturated NaCl. The water
layers were back extracted with 200 mL of CHCl₃. The
combined organics were dried with sodium sulfate and
30 evaporated to give 122 g of residue (approx. 90% product).
The residue was purified on a 3.5 kg silica gel column and
eluted using EtOAc/hexane(4:1). Pure product fractions were
evaporated to yield 96 g (84%). An additional 1.5 g was
recovered from later fractions.

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3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-
5 methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred
10 solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the
15 reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was
20 triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M)
25 in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400
30 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over

sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

5   2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was

10   evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica

15   column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

20   N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting

25   mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were

30   combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

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2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also
5 known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside
amidites] are prepared as described in the following
paragraphs. Adenosine, cytidine and guanosine nucleoside
amidites are prepared similarly to the thymidine (5-
methyluridine) except the exocyclic amines are protected
10 with a benzoyl moiety in the case of adenosine and cytidine
and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese,
15 Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g,
0.013eq, 0.0054mmol) were dissolved in dry pyridine (500
ml) at ambient temperature under an argon atmosphere and
with mechanical stirring. tert-Butyldiphenylchlorosilane
(125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one
20 portion. The reaction was stirred for 16 h at ambient
temperature. TLC (Rf 0.22, ethyl acetate) indicated a
complete reaction. The solution was concentrated under
reduced pressure to a thick oil. This was partitioned
between dichloromethane (1 L) and saturated sodium
25 bicarbonate (2x1 L) and brine (1 L). The organic layer was
dried over sodium sulfate and concentrated under reduced
pressure to a thick oil. The oil was dissolved in a 1:1
mixture of ethyl acetate and ethyl ether (600mL) and the
solution was cooled to
30 -10°C. The resulting crystalline product was collected by
filtration, washed with ethyl ether (3x200 mL) and dried
(40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC
and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622
5   mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were
10 added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for
15 ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions
20 used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl
25 acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting
30 material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-

methyluridine (20g, 36.98mmol) was mixed with
triphenylphosphine (11.63g, 44.36mmol) and N-
hydroxyphthalimide (7.24g, 44.36mmol). It was then dried
over P_2O_5 under high vacuum for two days at 40°C. The
5   reaction mixture was flushed with argon and dry THF
(369.8mL, Aldrich, sure seal bottle) was added to get a
clear solution. Diethyl-azodicarboxylate (6.98mL,
44.36mmol) was added dropwise to the reaction mixture. The
rate of addition is maintained such that resulting deep red
10   coloration is just discharged before adding the next drop.
After the addition was complete, the reaction was stirred
for 4 hrs. By that time TLC showed the completion of the
reaction (ethylacetate:hexane, 60:40). The solvent was
evaporated in vacuum. Residue obtained was placed on a
15   flash column and eluted with ethyl acetate:hexane (60:40),
to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-
butyldiphenylsilyl-5-methyluridine as white foam (21.819 g,
86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-
20   **formadoximinooxy)ethyl]-5-methyluridine**

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-
5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2
(4.5mL) and methylhydrazine (300mL, 4.64mmol) was added
dropwise at -10°C to 0°C. After 1 h the mixture was
25   filtered, the filtrate was washed with ice cold CH_2Cl_2 and
the combined organic phase was washed with water, brine and
dried over anhydrous Na_2SO_4. The solution was concentrated
to get 2'-O-(aminooxyethyl) thymidine, which was then
dissolved in MeOH (67.5mL). To this formaldehyde (20%
30   aqueous solution, w/w, 1.1 eq.) was added and the resulting
mixture was stirred for 1 h. Solvent was removed under
vacuum; residue chromatographed to get 5'-O-*tert*-
butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-
methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol)

5 was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the

10 reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na_2SO_4, evaporated to

15 dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol)

20 was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate

25 (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam

30 (14.6g, 80%).

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was

35 then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-

dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and
stirred at room temperature for 24 hrs. Reaction was
monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed
under vacuum and the residue placed on a flash column and
5   eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-
(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine
        2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg,
2.17mmol) was dried over P_2O_5 under high vacuum overnight at
10   40°C. It was then co-evaporated with anhydrous pyridine
(20mL). The residue obtained was dissolved in pyridine
(11mL) under argon atmosphere. 4-dimethylaminopyridine
(26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg,
2.60mmol) was added to the mixture and the reaction mixture
15   was stirred at room temperature until all of the starting
material disappeared. Pyridine was removed under vacuum
and the residue chromatographed and eluted with 10% MeOH in
CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-
2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

20   **5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-
         methyluridine-3'-[(2-cyanoethyl)-N,N-
         diisopropylphosphoramidite]**
        5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine
(1.08g, 1.67mmol) was co-evaporated with toluene (20mL).
25   To the residue N,N-diisopropylamine tetrazonide (0.29g,
1.67mmol) was added and dried over P_2O_5 under high vacuum
overnight at 40°C. Then the reaction mixture was dissolved
in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-
N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol)
30   was added. The reaction mixture was stirred at ambient
temperature for 4 hrs under inert atmosphere. The progress
of the reaction was monitored by TLC (hexane:ethyl acetate
1:1). The solvent was evaporated, then the residue was
dissolved in ethyl acetate (70mL) and washed with 5%

aqueous NaHCO₃ (40mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-

5 cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are

10 prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-

15 ### [(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-

20 (2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J.,

25 WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide

30 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-

diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH$_2$-O-CH$_2$-N(CH$_2$)$_2$, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetra-hydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O^2-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylamino-ethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8

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mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The combined CH_2Cl_2 layers
5   are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using $MeOH:CH_2Cl_2:Et_3N$ (20:1, v/v, with 1% triethylamine) gives the title compound.

10   **5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite**

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added
15   to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by
20   silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O)
25   oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard
30   oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and

deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.   Phosphinate oligonucleotides are prepared as

5   described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

10   3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent

15 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

20   3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by

25 reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

30 **Oligonucleoside Synthesis**

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedi-methylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino

35 linked oligonucleosides, also identified as amide-3 linked

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oligonucleosides, and methyleneaminocarbonyl linked oligo-
nucleosides, also identified as amide-4 linked oligonucleo-
sides, as well as mixed backbone compounds having, for
instance, alternating MMI and P=O or P=S linkages are

5 prepared as described in U.S. Patents 5,378,825, 5,386,023,
5,489,677, 5,602,240 and 5,610,289, all of which are
herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides
are prepared as described in U.S. Patents 5,264,562 and

10 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as
described in U.S. Patent 5,223,618, herein incorporated by
reference.

Example 4

15 **PNA Synthesis**

Peptide nucleic acids (PNAs) are prepared in
accordance with any of the various procedures referred to
in Peptide Nucleic Acids (PNA): Synthesis, Properties and
Potential Applications, *Bioorganic & Medicinal Chemistry*,

20 **1996**, **4**, 5-23. They may also be prepared in accordance
with U.S. Patents 5,539,082, 5,700,922, and 5,719,262,
herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

25 Chimeric oligonucleotides, oligonucleosides or mixed
oligonucleotides/oligonucleosides of the invention can be
of several different types. These include a first type
wherein the "gap" segment of linked nucleosides is
positioned between 5' and 3' "wing" segments of linked

30 nucleosides and a second "open end" type wherein the "gap"
segment is located at either the 3' or the 5' terminus of
the oligomeric compound. Oligonucleotides of the first type
are also known in the art as "gapmers" or gapped

oligonucleotides. Oligonucleotides of the second type are
also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

5   Chimeric oligonucleotides having 2'-O-alkyl
phosphorothioate and 2'-deoxy phosphorothioate oligo-
nucleotide segments are synthesized using an Applied
Biosystems automated DNA synthesizer Model 380B, as above.
Oligonucleotides are synthesized using the automated
10   synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphor-
amidite for the DNA portion and 5'-dimethoxytrityl-2'-O-
methyl-3'-O-phosphoramidite for 5' and 3' wings. The
standard synthesis cycle is modified by increasing the wait
step after the delivery of tetrazole and base to 600 s
15   repeated four times for RNA and twice for 2'-O-methyl. The
fully protected oligonucleotide is cleaved from the support
and the phosphate group is deprotected in 3:1
ammonia/ethanol at room temperature overnight then
lyophilized to dryness. Treatment in methanolic ammonia
20   for 24 hrs at room temperature is then done to deprotect
all bases and sample was again lyophilized to dryness. The
pellet is resuspended in 1M TBAF in THF for 24 hrs at room
temperature to deprotect the 2' positions. The reaction is
then quenched with 1M TEAA and the sample is then reduced
25   to 1/2 volume by rotovac before being desalted on a G25
size exclusion column. The oligo recovered is then
analyzed spectrophotometrically for yield and for purity by
capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

30

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxy-
ethyl)] chimeric phosphorothioate oligonucleotides were
prepared as per the procedure above for the 2'-O-methyl

chimeric oligonucleotide, with the substitution of 2'-O-
(methoxyethyl) amidites for the 2'-O-methyl amidites.

  [2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy

  Phosphorothioate]--[2'-O-(2-Methoxyethyl)

5   **Phosphodiester] Chimeric Oligonucleotides**

  [2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phos-
phorothioate]--[2'-O-(methoxyethyl) phosphodiester]
chimeric oligonucleotides are prepared as per the above
procedure for the 2'-O-methyl chimeric oligonucleotide with

10 the substitution of 2'-O-(methoxyethyl) amidites for the
2'-O-methyl amidites, oxidization with iodine to generate
the phosphodiester internucleotide linkages within the
wing portions of the chimeric structures and sulfurization
utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage

15 Reagent) to generate the phosphorothioate internucleotide
linkages for the center gap.

  Other chimeric oligonucleotides, chimeric oligonucleo-
sides and mixed chimeric oligonucleotides/oligonucleosides
are synthesized according to United States patent

20 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

  After cleavage from the controlled pore glass column
(Applied Biosystems) and deblocking in concentrated

25 ammonium hydroxide at 55°C for 18 hours, the
oligonucleotides or oligonucleosides are purified by
precipitation twice out of 0.5 M NaCl with 2.5 volumes
ethanol. Synthesized oligonucleotides were analyzed by
polyacrylamide gel electrophoresis on denaturing gels and

30 judged to be at least 85% full length material. The
relative amounts of phosphorothioate and phosphodiester
linkages obtained in synthesis were periodically checked by
[31]P nuclear magnetic resonance spectroscopy, and for some
studies oligonucleotides were purified by HPLC, as

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described by Chiang et al., *J. Biol. Chem.* 1991, *266*,
18162-18171. Results obtained with HPLC-purified material
were similar to those obtained with non-HPLC purified
material.

5   **Example 7**

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase
P(III) phosphoramidite chemistry on an automated
synthesizer capable of assembling 96 sequences

10 simultaneously in a standard 96 well format.
Phosphodiester internucleotide linkages were afforded by
oxidation with aqueous iodine. Phosphorothioate
internucleotide linkages were generated by sulfurization
utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage

15 Reagent) in anhydrous acetonitrile. Standard base-
protected beta-cyanoethyldiisopropyl phosphoramidites were
purchased from commercial vendors (e.g. PE-Applied
Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ).
Non-standard nucleosides are synthesized as per known

20 literature or patented methods. They are utilized as base
protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and
deprotected with concentrated NH$_4$OH at elevated temperature
(55-60°C) for 12-16 hours and the released product then

25 dried in vacuo. The dried product was then re-suspended in
sterile water to afford a master plate from which all
analytical and test plate samples are then diluted
utilizing robotic pipettors.

Example 8

30 **Oligonucleotide Analysis - 96 Well Plate Format**

The concentration of oligonucleotide in each well was
assessed by dilution of samples and UV absorption
spectroscopy. The full-length integrity of the individual
products was evaluated by capillary electrophoresis (CE) in

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either the 96 well format (Beckman P/ACE™ MDQ) or, for
individually prepared samples, on a commercial CE apparatus
(e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone
composition was confirmed by mass analysis of the compounds
5 utilizing electrospray-mass spectroscopy. All assay test
plates were diluted from the master plate using single and
multi-channel robotic pipettors. Plates were judged to be
acceptable if at least 85% of the compounds on the plate
were at least 85% full length.

10 **Example 9**

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic
acid expression can be tested in any of a variety of cell
types provided that the target nucleic acid is present at
15 measurable levels. This can be routinely determined using,
for example, PCR or Northern blot analysis. The following
four cell types are provided for illustrative purposes, but
other cell types can be routinely used.

20 T-24 cells:

The transitional cell bladder carcinoma cell line T-24
was obtained from the American Type Culture Collection
(ATCC) (Manassas, VA). T-24 cells were routinely cultured
in complete McCoy's 5A basal media (Gibco/Life
25 Technologies, Gaithersburg, MD) supplemented with 10% fetal
calf serum (Gibco/Life Technologies, Gaithersburg, MD),
penicillin 100 units per mL, and streptomycin 100
micrograms per mL (Gibco/Life Technologies, Gaithersburg,
MD). Cells were routinely passaged by trypsinization and
30 dilution when they reached 90% confluence. Cells were
seeded into 96-well plates (Falcon-Primaria #3872) at a
density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be
seeded onto 100 mm or other standard tissue culture plates

and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

5   The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life

10 Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

15

NHDF cells:

      Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium

20 (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

25   Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely

30 maintained for up to 10 passages as recommended by the supplier.

Treatment with antisense compounds:

      When cells reached 80% confluency, they were treated

35 with oligonucleotide. For cells grown in 96-well plates,

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wells were washed once with 200 μL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 μL of OPTI-MEM™-1 containing 3.75 μg/mL LIPOFECTIN™ (Gibco BRL) and the desired oligonucleotide at a final concentration of
5 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide treatment.

Example 10
Analysis of oligonucleotide inhibition of liver glycogen
10 phosphorylase expression

      Antisense modulation of liver glycogen phosphorylase expression can be assayed in a variety of ways known in the art. For example, liver glycogen phosphorylase mRNA levels can be quantitated by, e.g., Northern blot analysis,
15 competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, *F.M. et al.*, *Current Protocols in*
20 *Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley &
25 Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are
30 also known in the art.

      Liver glycogen phosphorylase protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell

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sorting (FACS). Antibodies directed to liver glycogen
phosphorylase can be identified and obtained from a variety
of sources, such as the MSRS catalog of antibodies (Aerie
Corporation, Birmingham, MI), or can be prepared via
5   conventional antibody generation methods. Methods for
preparation of polyclonal antisera are taught in, for
example, Ausubel, F.M. et al., *Current Protocols in
Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John
Wiley & Sons, Inc., **1997**. Preparation of monoclonal
10 antibodies is taught in, for example, Ausubel, F.M. et al.,
Current Protocols in Molecular Biology, Volume 2, pp.
11.4.1-11.11.5, John Wiley & Sons, Inc., **1997**.

Immunoprecipitation methods are standard in the art
and can be found at, for example, Ausubel, F.M. et al.,
15 *Current Protocols in Molecular Biology*, Volume 2, pp.
10.16.1-10.16.11, John Wiley & Sons, Inc., **1998**. Western
blot (immunoblot) analysis is standard in the art and can
be found at, for example, Ausubel, F.M. et al., *Current
Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-
20 10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked
immunosorbent assays (ELISA) are standard in the art and
can be found at, for example, Ausubel, F.M. et al., *Current
Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-
11.2.22, John Wiley & Sons, Inc., **1991**.

25 **Example 11**

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al.,
Clin. Chem., **1996**, *42*, 1758-1764. Other methods for
poly(A)+ mRNA isolation are taught in, for example,
30 Ausubel, F.M. et al., *Current Protocols in Molecular
Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons,
Inc., **1993**. Briefly, for cells grown on 96-well plates,
growth medium was removed from the cells and each well was
washed with 200 μL cold PBS. 60 μL lysis buffer (10 mM

Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μL of lysate was

5   transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove

10 excess wash buffer and then air-dried for 5 minutes. 60 μL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

15   Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

20   Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μL cold

25 PBS. 100 μL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a

30 QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of

35 the RNEASY 96™ plate and the vacuum applied for a period of

15 seconds. The Buffer RPE wash was then repeated and the
vacuum was applied for an additional 10 minutes. The plate
was then removed from the QIAVAC™ manifold and blotted dry
on paper towels. The plate was then re-attached to the

5   QIAVAC™ manifold fitted with a collection tube rack
containing 1.2 mL collection tubes. RNA was then eluted by
pipetting 60 μL water into each well, incubating 1 minute,
and then applying the vacuum for 30 seconds. The elution
step was repeated with an additional 60 μL water.

10   The repetitive pipetting and elution steps may be
automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc.,
Valencia CA). Essentially after lysing of the cells on the
culture plate, the plate is transferred to the robot deck
where the pipetting, DNase treatment and elution steps are

15   carried out.

Example 13

**Real-time Quantitative PCR Analysis of liver glycogen
phosphorylase mRNA Levels**

         Quantitation of liver glycogen phosphorylase mRNA
20   levels was determined by real-time quantitative PCR using
the ABI PRISM™ 7700 Sequence Detection System (PE-Applied
Biosystems, Foster City, CA) according to manufacturer's
instructions. This is a closed-tube, non-gel-based,
fluorescence detection system which allows high-throughput

25   quantitation of polymerase chain reaction (PCR) products in
real-time. As opposed to standard PCR, in which
amplification products are quantitated after the PCR is
completed, products in real-time quantitative PCR are
quantitated as they accumulate. This is accomplished by

30   including in the PCR reaction an oligonucleotide probe that
anneals specifically between the forward and reverse PCR
primers, and contains two fluorescent dyes. A reporter dye
(e.g., JOE or FAM, obtained from either Operon Technologies
Inc., Alameda, CA or PE-Applied Biosystems, Foster City,

35   CA) is attached to the 5' end of the probe and a quencher

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dye (e.g., TAMRA, obtained from either Operon Technologies
Inc., Alameda, CA or PE-Applied Biosystems, Foster City,
CA) is attached to the 3' end of the probe. When the probe
and dyes are intact, reporter dye emission is quenched by
5 the proximity of the 3' quencher dye. During
amplification, annealing of the probe to the target
sequence creates a substrate that can be cleaved by the 5'-
exonuclease activity of Taq polymerase. During the
extension phase of the PCR amplification cycle, cleavage of
10 the probe by Taq polymerase releases the reporter dye from
the remainder of the probe (and hence from the quencher
moiety) and a sequence-specific fluorescent signal is
generated. With each cycle, additional reporter dye
molecules are cleaved from their respective probes, and the
15 fluorescence intensity is monitored at regular intervals by
laser optics built into the ABI PRISM™ 7700 Sequence
Detection System. In each assay, a series of parallel
reactions containing serial dilutions of mRNA from
untreated control samples generates a standard curve that
20 is used to quantitate the percent inhibition after
antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems,
Foster City, CA. RT-PCR reactions were carried out by
adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM
25 $MgCl_2$, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP,
100 nM each of forward primer, reverse primer, and probe,
20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™, and
12.5 Units MuLV reverse transcriptase) to 96 well plates
containing 25 µL poly(A) mRNA solution. The RT reaction
30 was carried out by incubation for 30 minutes at 48°C.
Following a 10 minute incubation at 95°C to activate the
AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were
carried out: 95°C for 15 seconds (denaturation) followed by
60°C for 1.5 minutes (annealing/extension). Liver glycogen
35 phosphorylase probes and primers were designed to hybridize

to the human liver glycogen phosphorylase sequence, using
published sequence information (GenBank accession number
M14636, incorporated herein as SEQ ID NO:1).

    For liver glycogen phosphorylase the PCR primers were:
5 forward primer: CATGGGCCGAACATTACAGAA (SEQ ID NO: 2)
reverse primer: CAAGACCACCATTGCCAAGTC (SEQ ID NO: 3) and
the PCR probe was: FAM-CTGTGATGAGGCCATTTACCAGCTTGG-TAMRA
(SEQ ID NO: 4) where FAM (PE-Applied Biosystems, Foster
City, CA) is the fluorescent reporter dye) and TAMRA (PE-
10 Applied Biosystems, Foster City, CA) is the quencher dye.

    For GAPDH the PCR primers were:
forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 5)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 6)and the
PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ
15 ID NO: 7) where JOE (PE-Applied Biosystems, Foster City,
CA) is the fluorescent reporter dye) and TAMRA (PE-Applied
Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of liver glycogen phosphorylase mRNA
20 **levels**

    Eighteen hours after antisense treatment, cell
monolayers were washed twice with cold PBS and lysed in 1
mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA
was prepared following manufacturer's recommended
25 protocols. Twenty micrograms of total RNA was fractionated
by electrophoresis through 1.2% agarose gels containing
1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc.
Solon, OH). RNA was transferred from the gel to HYBOND™-N+
nylon membranes (Amersham Pharmacia Biotech, Piscataway,
30 NJ) by overnight capillary transfer using a
Northern/Southern Transfer buffer system (TEL-TEST "B"
Inc., Friendswood, TX). RNA transfer was confirmed by UV
visualization. Membranes were fixed by UV cross-linking
using a STRATALINKER™ UV Crosslinker 2400 (Stratagene,
35 Inc, La Jolla, CA).

Membranes were probed using QUICKHYB™ hybridization
solution (Stratagene, La Jolla, CA) using manufacturer's
recommendations for stringent conditions with a liver
glycogen phosphorylase specific probe prepared by PCR using
5 the forward primer CATGGGCCGAACATTACAGAA (SEQ ID NO: 2) and
the reverse primer CAAGACCACCATTGCCAAGTC (SEQ ID NO: 3).
To normalize for variations in loading and transfer
efficiency membranes were stripped and probed for
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA
10 (Clontech, Palo Alto, CA). Hybridized membranes were
visualized and quantitated using a PHOSPHORIMAGER™ and
IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale,
CA). Data was normalized to GAPDH levels in untreated
controls.

15 **Example 15**

Antisense inhibition of liver glycogen phosphorylase
expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of
oligonucleotides were designed to target different regions
20 of the human liver glycogen phosphorylase RNA, using
published sequences (GenBank accession number M14636,
incorporated herein as SEQ ID NO: 1). The oligonucleotides
are shown in Table 1. Target sites are indicated by
nucleotide numbers, as given in the sequence source
25 reference (Genbank accession no. M14636), to which the
oligonucleotide binds. All compounds in Table 1 are
oligodeoxynucleotides with phosphorothioate backbones
(internucleoside linkages) throughout. The compounds were
analyzed for effect on liver glycogen phosphorylase mRNA
30 levels by quantitative real-time PCR as described in other
examples herein. Data are averages from two experiments.
If present, "N.D." indicates "no data".

Table 1

Inhibition of liver glycogen phosphorylase mRNA levels by
phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
104049	5' UTR	9	ccgccccgccgcgccaggag	0	8
104050	5' UTR	71	cgggctgcgcagagagctgg	13	9
104051	Start Codon	109	cagcggttcgcccatggctg	2	10
104052	Start Codon	114	tctgtcagcggttcgcccat	31	11
104053	Coding	172	tgccacgttctccacgccca	82	12
104054	Coding	220	cttgaccagcgtgaagtgca	52	13
104055	Coding	260	gcgcgaagtagtagtcgcgg	20	14
104056	Coding	299	tccagcgccccaccaggtgg	56	15
104057	Coding	372	cccatgtaaaattccagaga	56	16
104058	Coding	415	attttgcagaccgaggttga	69	17
104059	Coding	460	ctcttctatatccaatccaa	37	18
104060	Coding	523	gcaggcagcaagtctcccaa	71	19
104061	Coding	571	gccgtatccataggctgcaa	0	20
104062	Coding	625	tacctgccatccatctcgga	85	21
104063	Coding	678	gggcgggacttctcccaagg	21	22
104064	Coding	734	tcccggtgttggtgtgttct	37	23
104065	Coding	817	ggtgttgacagtgttattca	44	24
104066	Coding	877	tccaacattaaagtctctga	38	25
104067	Coding	949	attgtcattgggatagagga	51	26
104068	Coding	1052	tggagccaaacttggaggct	68	27
104069	Coding	1206	ttctggttgagctcccatgc	48	28
104070	Coding	1263	acgggccagcgctccagggc	53	29
104071	Coding	1327	atgcttctgatttatctcat	51	30
104072	Coding	1389	atcagagacatccttctcag	67	31
104073	Coding	1453	cacagcatgggaaccgacaa	85	32
104074	Coding	1510	gaagtccttgaatactttag	60	33
104075	Coding	1598	ctgcaagtcctgggttgcag	51	34
104076	Coding	1678	atcacccaggaagctgtgga	69	35
104077	Coding	1778	aggatgggttgatcttcact	61	36
104078	Coding	1837	acagttcaagagctgtcgct	61	37
104079	Coding	1925	cagctttaccaccaatgata	71	38
104080	Coding	2009	ttccaaccatagggtcattg	73	39
104081	Coding	2080	atctgtggctggaatgactt	21	40
104082	Coding	2135	tcatattgcctgtccccgag	82	41
104083	Coding	2189	ccacattggccccatccatg	81	42
104084	Coding	2241	atgctcatgccaaagatgaa	2	43
104085	Coding	2291	agtattcttttgcctcgtac	79	44
104086	Coding	2337	ttgtcaatttgatcaatgac	38	45

```
104087 Coding  2403   tcatgataaaatagcatgtt        0        46
104088 3' UTR   2758   ccccattcccagagatactc        68       47
```

        As shown in Table 1, SEQ ID NOs 11, 12, 13, 15, 16,
5 17, 18, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33,
    34, 35, 36, 37, 38, 39, 41, 42, 44, 45 and 47 demonstrated
    at least 30% inhibition of liver glycogen phosphorylase
    expression in this assay and are therefore preferred.
    Example 16:
10 **Antisense inhibition of liver glycogen phosphorylase**
    expression- phosphorothioate 2'-MOE gapmer oligonucleotides
        In accordance with the present invention, a second
    series of oligonucleotides targeted to human liver glycogen
    phosphorylase were synthesized. The oligonucleotide
15 sequences are shown in Table 2. Target sites are indicated
    by nucleotide numbers, as given in the sequence source
    reference (Genbank accession no. M14636), to which the
    oligonucleotide binds.
        All compounds in Table 2 are chimeric oligonucleotides
20 ("gapmers") 20 nucleotides in length, composed of a central
    "gap" region consisting of ten 2'-deoxynucleotides, which
    is flanked on both sides (5' and 3' directions) by five-
    nucleotide "wings". The wings are composed of 2'-
    methoxyethyl (2'-MOE)nucleotides. The internucleoside
25 (backbone) linkages are phosphorothioate (P=S) throughout
    the oligonucleotide. Cytidine residues in the 2'-MOE wings
    are 5-methylcytidines.
        Data were obtained by real-time quantitative PCR as
    described in other examples herein and are averaged from
30 two experiments. If present, "N.D." indicates "no data".

Table 2

Inhibition of liver glycogen phosphorylase mRNA levels by
chimeric phosphorothioate oligonucleotides having 2'-MOE
wings and a deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
104089	5' UTR	9	ccgccccgccgcgccaggag	0	8
104090	5' UTR	71	cgggctgcgcagagagctgg	61	9
104091	Start Codon	109	cagcggttcgcccatggctg	20	10
104092	Start Codon	114	tctgtcagcggttcgcccat	23	11
104093	Coding	172	tgccacgttctccacgccca	90	12
104094	Coding	220	cttgaccagcgtgaagtgca	88	13
104095	Coding	260	gcgcgaagtagtagtcgcgg	76	14
104096	Coding	299	tccagcgccccaccaggtgg	87	15
104097	Coding	372	cccatgtaaaattccagaga	98	16
104098	Coding	415	attttgcagaccgaggttga	94	17
104099	Coding	460	ctcttctatatccaatccaa	71	18
104100	Coding	523	gcaggcagcaagtctcccaa	57	19
104101	Coding	571	gccgtatccataggctgcaa	97	20
104102	Coding	625	tacctgccatccatctcgga	89	21
104103	Coding	678	gggcgggacttctcccaagg	79	22
104104	Coding	734	tcccggtgttggtgtgttct	89	23
104105	Coding	817	ggtgttgacagtgttattca	95	24
104106	Coding	877	tccaacattaaagtctctga	77	25
104107	Coding	949	attgtcattgggatagagga	92	26
104108	Coding	1052	tggagccaaacttggaggct	77	27
104109	Coding	1206	ttctggttgagctcccatgc	48	28
104110	Coding	1263	acgggccagcgctccagggc	82	29
104111	Coding	1327	atgcttctgatttatctcat	83	30
104112	Coding	1389	atcagagacatccttctcag	96	31
104113	Coding	1453	cacagcatgggaaccgacaa	88	32
104114	Coding	1510	gaagtccttgaatactttag	86	33
104115	Coding	1598	ctgcaagtcctgggttgcag	74	34
104116	Coding	1678	atcacccaggaagctgtgga	70	35
104117	Coding	1778	aggatgggttgatcttcact	70	36
104118	Coding	1837	acagttcaagagctgtcgct	85	37
104119	Coding	1925	cagctttaccaccaatgata	58	38
104120	Coding	2009	ttccaaccatagggtcattg	86	39
104121	Coding	2080	atctgtggctggaatgactt	40	40
104122	Coding	2135	tcatattgcctgtccccgag	49	41
104123	Coding	2189	ccacattggccccatccatg	90	42
104124	Coding	2241	atgctcatgccaaagatgaa	53	43

104125 Coding	2291	agtattcttttgcctcgtac	86	44
104126 Coding	2337	ttgtcaatttgatcaatgac	73	45
104127 Coding	2403	tcatgataaaatagcatgtt	71	46
104128 3' UTR	2758	ccccattcccagagatactc	82	47

5

As shown in Table 2, SEQ ID NOs 9, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46 and 47 demonstrated at least 30% inhibition of liver

10 glycogen phosphorylase expression in this experiment and are therefore preferred.

Example 17

Western blot analysis of liver glycogen phosphorylase protein levels

15   Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for

20 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to liver glycogen phosphorylase is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a

25 PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

What is claimed is:

1.   An antisense compound 8 to 30 nucleobases in length targeted to a nucleic acid molecule encoding human liver glycogen phosphorylase, wherein said antisense compound specifically hybridizes with and inhibits the expression of human liver glycogen phosphorylase.

2.   The antisense compound of claim 1 which is an antisense oligonucleotide.

3.   The antisense compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46 or 47.

4.   The antisense compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 12, 13, 15, 16, 17, 18, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 41, 42, 44, 45 or 47.

5.   The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

6.   The antisense compound of claim 5 wherein the modified internucleoside linkage is a phosphorothioate linkage.

7.   The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

8.   The antisense compound of claim 7 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

9.   The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

10.   The antisense compound of claim 9 wherein the modified nucleobase is a 5-methylcytosine.

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11. The antisense compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

12. A composition comprising the antisense compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

14. The composition of claim 12 wherein the antisense compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of liver glycogen phosphorylase in human cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 1 so that expression of liver glycogen phosphorylase is inhibited.

16. A method of treating a human having a disease or condition associated with liver glycogen phosphorylase comprising administering to said animal a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that expression of liver glycogen phosphorylase is inhibited.

17. The method of claim 16 wherein the disease or condition is diabetes.

18. The method of claim 17 wherein the disease or condition is diabetes type II.

SEQUENCE LISTING

<110> Brett P. Monia
      Lex M. Cowsert
      ISIS PHARMACEUTICALS, Inc.

<120> ANTISENSE MODULATION OF LIVER GLYCOGEN PHOSPHORYLASE EXPRESSION

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<150> US 09/357,071
<151> 1999-07-19

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                                                               Met
                                                                 1

ggc gaa ccg ctg aca gac cag gag aag cgg cgg cag atc agc atc cgc   164
Gly Glu Pro Leu Thr Asp Gln Glu Lys Arg Arg Gln Ile Ser Ile Arg
            5   10   15

ggc atc gtg ggc gtg gag aac gtg gca gag ctg aag aag agt ttc aac   212
Gly Ile Val Gly Val Glu Asn Val Ala Glu Leu Lys Lys Ser Phe Asn
        20   25   30

cgg cac ctg cac ttc acg ctg gtc aag gac cgc aac gtg gcc acc acc   260
Arg His Leu His Phe Thr Leu Val Lys Asp Arg Asn Val Ala Thr Thr
    35   40   45

cgc gac tac tac ttc gcg ctg gcg cac acg gtg cgg gac cac ctg gtg   308
Arg Asp Tyr Tyr Phe Ala Leu Ala His Thr Val Arg Asp His Leu Val
50   55   60   65

ggg cgc tgg atc cgc acg cag cag cac tac tac gac aag tgc ccc aag   356
Gly Arg Trp Ile Arg Thr Gln Gln His Tyr Tyr Asp Lys Cys Pro Lys
                70   75   80

agg gaa tat tac ctc tct ctg gaa ttt tac atg ggc cga aca tta cag   404
Arg Glu Tyr Tyr Leu Ser Leu Glu Phe Tyr Met Gly Arg Thr Leu Gln
            85   90   95

aac acc atg atc aac ctc ggt ctg caa aat gcc tgt gat gag gcc att   452
Asn Thr Met Ile Asn Leu Gly Leu Gln Asn Ala Cys Asp Glu Ala Ile
        100   105   110

tac cag ctt gga ttg gat ata gaa gag tta gaa gaa att gaa gaa gat   500
Tyr Gln Leu Gly Leu Asp Ile Glu Glu Leu Glu Glu Ile Glu Glu Asp
    115   120   125

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gct gga ctt ggc aat ggt ggt ctt ggg aga ctt gct gcc tgc ttc ttg        548
Ala Gly Leu Gly Asn Gly Gly Leu Gly Arg Leu Ala Ala Cys Phe Leu
130             135                 140                 145

gat tcc atg gca acc ctg gga ctt gca gcc tat gga tac ggc att cgg        596
Asp Ser Met Ala Thr Leu Gly Leu Ala Ala Tyr Gly Tyr Gly Ile Arg
                150                 155                 160

tat gaa tat ggg att ttc aat cag aag atc cga gat gga tgg cag gta        644
Tyr Glu Tyr Gly Ile Phe Asn Gln Lys Ile Arg Asp Gly Trp Gln Val
            165                 170                 175

gaa gaa gca gat gat tgg ctc aga tat gga aac cct tgg gag aag tcc        692
Glu Glu Ala Asp Asp Trp Leu Arg Tyr Gly Asn Pro Trp Glu Lys Ser
            180                 185                 190

cgc cca gaa ttc atg ctg cct gtg cac ttc tat gga aaa gta gaa cac        740
Arg Pro Glu Phe Met Leu Pro Val His Phe Tyr Gly Lys Val Glu His
        195                 200                 205

acc aac acc ggg acc aag tgg att gac act caa gtg gtc ctg gct ctg        788
Thr Asn Thr Gly Thr Lys Trp Ile Asp Thr Gln Val Val Leu Ala Leu
210             215                 220                 225

cca tat gac acc ccc gag ccc ggc tac atg aat aac act gtc aac acc        836
Pro Tyr Asp Thr Pro Glu Pro Gly Tyr Met Asn Asn Thr Val Asn Thr
                230                 235                 240

atg cgc ctc tgg tct gct cgg gca cca aat gac ttt aac ctc aga gac        884
Met Arg Leu Trp Ser Ala Arg Ala Pro Asn Asp Phe Asn Leu Arg Asp
                245                 250                 255

ttt aat gtt gga gac tac att cag gct gtg ctg gac cga aac ctg gcc        932
Phe Asn Val Gly Asp Tyr Ile Gln Ala Val Leu Asp Arg Asn Leu Ala
            260                 265                 270

gag aac atc tcc cgg gtc ctc tat ccc aat gac aat ttt ttt gaa ggg        980
Glu Asn Ile Ser Arg Val Leu Tyr Pro Asn Asp Asn Phe Phe Glu Gly
        275                 280                 285

aag gag cta aga ttg aag cag gaa tac ttt gtg gtg gct gca acc ttg        1028
Lys Glu Leu Arg Leu Lys Gln Glu Tyr Phe Val Val Ala Ala Thr Leu
290             295                 300                 305

caa gat atc atc cgc cgt ttc aaa gcc tcc aag ttt ggc tcc acc cgt        1076
Gln Asp Ile Ile Arg Arg Phe Lys Ala Ser Lys Phe Gly Ser Thr Arg
                310                 315                 320

ggt caa gga act gtg ttt gat gcc ttc ccg gat cag gtg gcc atc cag        1124
Gly Gln Gly Thr Val Phe Asp Ala Phe Pro Asp Gln Val Ala Ile Gln
                325                 330                 335

ctg aat gat act cac cct cgc atc gcg atc cct gag ctg atg agg att        1172
Leu Asn Asp Thr His Pro Arg Ile Ala Ile Pro Glu Leu Met Arg Ile
            340                 345                 350

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Phe Val Asp Ile Glu Lys Leu Pro Trp Ser Lys Ala Trp Glu Leu Asn
            355                 360                 365

cag aag acc ttc gcc tac acc aac cac aca gtg ctc ccg gaa gcc ctg        1268
Gln Lys Thr Phe Ala Tyr Thr Asn His Thr Val Leu Pro Glu Ala Leu
370             375                 380                 385
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gag cgc tgg ccc gtg gac ctg gtg gag aag ctg ctc cct cga cat ttg      1316
Glu Arg Trp Pro Val Asp Leu Val Glu Lys Leu Leu Pro Arg His Leu
            390                 395                 400

gaa atc att tat gag ata aat cag aag cat tta gat aga att gtg gcc      1364
Glu Ile Ile Tyr Glu Ile Asn Gln Lys His Leu Asp Arg Ile Val Ala
            405                 410                 415

ttg ttt cct aaa gat gtg gac cct ctg aga agg atg tct ctg ata gaa      1412
Leu Phe Pro Lys Asp Val Asp Pro Leu Arg Arg Met Ser Leu Ile Glu
            420                 425                 430

gag gaa gga agc aaa agg atc aac atg gcc cat ctc tgc att gtc ggt      1460
Glu Glu Gly Ser Lys Arg Ile Asn Met Ala His Leu Cys Ile Val Gly
        435                 440                 445

tcc cat gct gtg aat ggc gtg gct aaa atc cac tca gac atc gtg aag      1508
Ser His Ala Val Asn Gly Val Ala Lys Ile His Ser Asp Ile Val Lys
450                 455                 460                 465

act aaa gta ttc aag gac ttc agt gag cta gaa cct gac aag ttt cag      1556
Thr Lys Val Phe Lys Asp Phe Ser Glu Leu Glu Pro Asp Lys Phe Gln
                470                 475                 480

aat aaa acc aat ggg atc act cca agg cgc tgg ctc cta ctc tgc aac      1604
Asn Lys Thr Asn Gly Ile Thr Pro Arg Arg Trp Leu Leu Leu Cys Asn
            485                 490                 495

cca gga ctt gca gag ctc ata gca gag aaa att gga gaa gac tat gtg      1652
Pro Gly Leu Ala Glu Leu Ile Ala Glu Lys Ile Gly Glu Asp Tyr Val
            500                 505                 510

aaa gac ctg agc cag ctg acg aag ctc cac agc ttc ctg ggt gat gat      1700
Lys Asp Leu Ser Gln Leu Thr Lys Leu His Ser Phe Leu Gly Asp Asp
        515                 520                 525

gtc ttc ctc cgg gaa ctc gcc aag gtg aag cag gag aat aag ctg aag      1748
Val Phe Leu Arg Glu Leu Ala Lys Val Lys Gln Glu Asn Lys Leu Lys
530                 535                 540                 545

ttt tct cag ttc ctg gag acg gag tac aaa gtg aag atc aac cca tcc      1796
Phe Ser Gln Phe Leu Glu Thr Glu Tyr Lys Val Lys Ile Asn Pro Ser
                550                 555                 560

tcc atg ttt gat gtc cag gtg aag agg ata cat gag tac aag cga cag      1844
Ser Met Phe Asp Val Gln Val Lys Arg Ile His Glu Tyr Lys Arg Gln
            565                 570                 575

ctc ttg aac tgt ctg cat gtg atc acg atg tac aac cgc att aag aaa      1892
Leu Leu Asn Cys Leu His Val Ile Thr Met Tyr Asn Arg Ile Lys Lys
            580                 585                 590

gac cct aag aag tta ttc gtg cca agg aca gtt atc att ggt ggt aaa      1940
Asp Pro Lys Lys Leu Phe Val Pro Arg Thr Val Ile Ile Gly Gly Lys
        595                 600                 605

gct gcc cca gga tat cac atg gcc aaa atg atc ata aag ctg atc act      1988
Ala Ala Pro Gly Tyr His Met Ala Lys Met Ile Ile Lys Leu Ile Thr
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tca gtg gca gat gtg gtg aac aat gac cct atg gtt gga agc aag ttg      2036
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3

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Ile Pro Ala Thr Asp Leu Ser Glu Gln Ile Ser Thr Ala Gly Thr Glu
            660             665             670

gcc tcg ggg aca ggc aat atg aag ttc atg cta aat ggg gcc cta act     2180
Ala Ser Gly Thr Gly Asn Met Lys Phe Met Leu Asn Gly Ala Leu Thr
            675             680             685

atc ggg acc atg gat ggg gcc aat gtg gaa atg gca gaa gaa gct ggg     2228
Ile Gly Thr Met Asp Gly Ala Asn Val Glu Met Ala Glu Glu Ala Gly
690             695             700             705

gaa gag aac ctg ttc atc ttt ggc atg agc ata gat gat gtg gct gct     2276
Glu Glu Asn Leu Phe Ile Phe Gly Met Ser Ile Asp Asp Val Ala Ala
            710             715             720

ttg gac aag aaa ggg tac gag gca aaa gaa tac tat gag gca ctt cca     2324
Leu Asp Lys Lys Gly Tyr Glu Ala Lys Glu Tyr Tyr Glu Ala Leu Pro
            725             730             735

gag ctg aag ctg gtc att gat caa att gac aat ggc ttt ttt tct ccc     2372
Glu Leu Lys Leu Val Ile Asp Gln Ile Asp Asn Gly Phe Phe Ser Pro
            740             745             750

aag cag cct gac ctc ttc aaa gat atc atc aac atg cta ttt tat cat     2420
Lys Gln Pro Asp Leu Phe Lys Asp Ile Ile Asn Met Leu Phe Tyr His
            755             760             765

gac agg ttt aaa gtc ttt gca gac tac gaa gcc tat gtc aag tgt caa     2468
Asp Arg Phe Lys Val Phe Ala Asp Tyr Glu Ala Tyr Val Lys Cys Gln
770             775             780             785

gat aaa gtg agt cag ctg tac atg aat cca aag gcc tgg aac aca atg     2516
Asp Lys Val Ser Gln Leu Tyr Met Asn Pro Lys Ala Trp Asn Thr Met
            790             795             800

gta ctc aaa aac ata gct gcc tcg ggg aaa ttc tcc agt gac cga aca     2564
Val Leu Lys Asn Ile Ala Ala Ser Gly Lys Phe Ser Ser Asp Arg Thr
            805             810             815

att aaa gaa tat gcc caa aac atc tgg aac gtg gaa cct tca gat cta     2612
Ile Lys Glu Tyr Ala Gln Asn Ile Trp Asn Val Glu Pro Ser Asp Leu
            820             825             830

aag att tct cta tcc aat gaa tct aac aaa gtc aat gga aat tga        2657
Lys Ile Ser Leu Ser Asn Glu Ser Asn Lys Val Asn Gly Asn
835             840             845

actctacaat gtctctagaa aacatagctt cttactgaac ttgaacattt ttacaacatt  2717

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<210> 47
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INTERNATIONAL SEARCH REPORT	International application No. PCT/US00/19019

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :C12N 15/00, 15/11; C12Q 1/68; A61K 48/00
US CL   : 435/6, 366, 375, 91.1; 536/23.1, 24.3, 24.5; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/6, 366, 375, 91.1; 536/23.1, 24.3, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRANCH, A.D. A good antisense molecule is hard to find. TIBS February 1998, Vol. 23, pages 45-50, see entire document.	16-18NO
Y	CROOKE, S.T. 'Basic Principles of Antisense Therapeutics.' In: Antisense Research and Application. Edited by S. T. Crooke. 1998, p. 3-49, see entire document.	1-2, 5-11, 15
Y	UHLMANN et al. Antisense Oligonucleotides: A New Therapeutic Principle. Chemical Reviews. June 1990, Vol. 90, No. 4, pages 543-584, see entire document.	1-2, 5-11, 15

☒	Further documents are listed in the continuation of Box C.	☐	See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 15 SEPTEMBER 2000	Date of mailing of the international search report 15 DEC 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DELLA MAE COLLINS PARALEGAL SPECIALIST MARY SCHMIDT TECHNOLOGY CENTER 1600 Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MILNER et al., "Selecting effective antisense reagents on combinatorial oligonucleotide arrays," Nature Biotechnology. June 1997, Vol. 15, pages 537-541, see entire document.	1-2, 5-11, 15 1-2
Y	NEWGARD et al. "The Polymorphic Locus for Glycogen Storage Disease VI (Liver Glycogen Phosphorylase) Maps to Chromosome 14," Am. J. Hum. Genetics. 1987, Vol. 40, pages 351-364.	1-2, 5-11, 15
Y	HOOVER et al., "Indole-2-carboxamide inhibitors of Human Liver Glycogen Phosphorylase," J. Med. chem. 1998, Vol. 41, pages 2934-2938, see entire document.	1-2, 5-11, 15